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Helicobacter pylori induces but survives the extracellular release of oxygen radicals from professional phagocytes using its catalase activity

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Summary

***Helicobacter pylori* can colonize the gastric epithelium of humans, leading to the induction of an intense inflammatory response with the infiltration of mainly polymorphonuclear leucocytes (PMNs) and monocytes. These professional phagocytes appear to be a primary cause of the damage to surface epithelial layers, and probably contribute to the pathogenesis associated with persistent *H. pylori* infections. We have shown previously that *H. pylori* adheres to professional phagocytes, but is not engulfed efficiently, suggesting an antiphagocytic escape mechanism that is dependent on the pathogen's type IV secretion system. Here, we show that *H. pylori* induces the generation and extracellular release of oxygen metabolites as a consequence of its attachment to phagocytic cells, but is capable of surviving this response. The catalase activity of *H. pylori* is apparently essential for survival at the phagocytes' cell surface. Opsonization of *H. pylori* leads to an increased burst, and the inhibition of bacterial protein synthesis to a decreased one. Ca^{2+} concentration, cytoskeleton rearrangement and protein kinase C (PKC) are involved in the *H. pylori*-induced oxidative burst in both monocytes and PMNs. This survival phenomenon has important implications for both the persistence of this important pathogen and the host tissue damage that accompanies persistent *H. pylori* infection.**

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Introduction

Helicobacter pylori is a spiral, Gram-negative bacterial pathogen (Marshall and Warren, 1983) that can colonize the gastric epithelium of humans and other primates. Persistent *H. pylori* infections are closely associated with the development of active chronic gastritis, duodenitis, gastric or duodenal ulcers and gastric carcinomas (Wallace, 1991), and these symptoms can be resolved by antibiotic treatment to kill the bacteria (Megraud and Lamouliatte, 1992). Lymphocytes, macrophages and polymorphonuclear neutrophils (PMNs) are recruited to the site of infection, and an immune response is induced, which also results in the production of antibodies (Andersen *et al*., 1992). Despite this strong inflammatory response, *H. pylori* eradication and disease recovery are not observed without antibiotic treatment (Fiocca *et al*., 1994), suggesting that the pathogen has evolved effective mechanisms to resist the host's immune response (Zevering *et al*., 1999).

Although the immune response induced by *H. pylori* appears to be ineffective against these bacteria, it does have important implications for the onset of disease. The severity of gastric mucosal damage observed during chronic antral gastritis is directly correlated with the extent of phagocytic infiltration (Kozol *et al*., 1991; Yoshida *et al*., 1993). We have demonstrated recently that *H. pylori* actively adheres to PMNs and monocytes, but blocks its own phagocytosis by these professional phagocytes (Ramarao *et al*., 2000). This is an active process, as the inhibition of bacterial protein synthesis results in the uptake of *H. pylori*. The antiphagocytic activity also appears to occur via a global inhibition of phagocytosis pathways, as co-infection with *H. pylori* also blocks the uptake of *Neisseria gonorrhoeae* and latex bead engulfment by these cells.

Although this mechanism implies obvious benefits for the bacteria's ability to establish a persistent infection, professional phagocytes have the capacity to release toxic metabolites into the extracellular milieu in an attempt to kill the parasite during such a 'frustrated phagocytosis' (Bergstrand, 1990). The most obvious response to contact with an activating stimulus is the respiratory burst, a process that is characterized by an increase in oxygen consumption, activation of a metabolic hexose

monophosphate shunt and the generation and release of chemically reactive oxygen metabolites, including superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl radicals (DeChatelet *et al.*, 1982). Some bacterial pathogens have evolved strategies for dealing with this potent bactericidal response. For instance, *N. gonorrhoeae* Opa adhesins mediate attachment to PMNs and monocytes by a process that leads to a rapid phagocytosis of the bacteria and an enhanced oxidative response (Gray-Owen *et al.*, 1997a). Although the benefit of this interaction to gonococci is uncertain, it may represent a mechanism by which the engulfed organism can force the phagocyte to expend large amounts of energy and, thereby, provide a survival advantage to the infecting population. In contrast, *Yersinia enterocolitica* actively blocks its own engulfment and the phagocytic oxidative response (Lian *et al.*, 1987), allowing the pathogen to stay at the surface of these otherwise potently bactericidal cells.

In this study, we have characterized the oxidative response of professional phagocytes to bound *H. pylori* and assessed the viability of the bacteria that remain adherent at their surface. We found that an amplified oxidative burst occurs in response to *H. pylori* binding, but that the bound bacteria survive this response. This survival was not caused by a lack of exposure to generated oxygen metabolites, as we found that the vast majority of peroxide was released into the culture supernatant, and co-infection experiments demonstrated an effective phagocytic killing of surface-bound *N. gonorrhoeae*. In addition to the obvious advantage inherent in *H. pylori's* ability to block phagocytosis and survive the phagocyte's oxidative killing response, the intense release of oxidative metabolites into the extracellular milieu also has major implications in our understanding of the pathological consequences of this important pathogen.

Results

H. pylori *induces an oxidative burst by professional phagocytes*

In previous studies, we demonstrated that *H. pylori* actively blocks its own phagocytosis by professional phagocytes (Ramarao *et al.*, 2000). In order to understand how the phagocytes respond to these extracellularly associated bacteria, we tested whether an oxidative response was induced by *H. pylori* infection. Resting phagocytes display only basal chemiluminescence (CL) in the presence of luminol, whereas the addition of phorbol myristate acetate (PMA), a direct activator of protein kinase C (PKC) and a potent agonist of the NADPH oxidase (Downey, 1998), or of latex beads (not shown) resulted in rapid and intense CL responses (Fig. 1A). The addition of *H. pylori* to primary monocytes or PMNs triggers a relatively weaker oxidative burst, albeit one that is higher than that triggered by *N. gonorrhoeae* expressing the well-described CD66 receptor-specific Opa_{52} adhesin (Fig. 1A; Dehio *et al.*, 1998). In contrast, *Y. enterocolitica,* grown at 37°C to allow expression of its

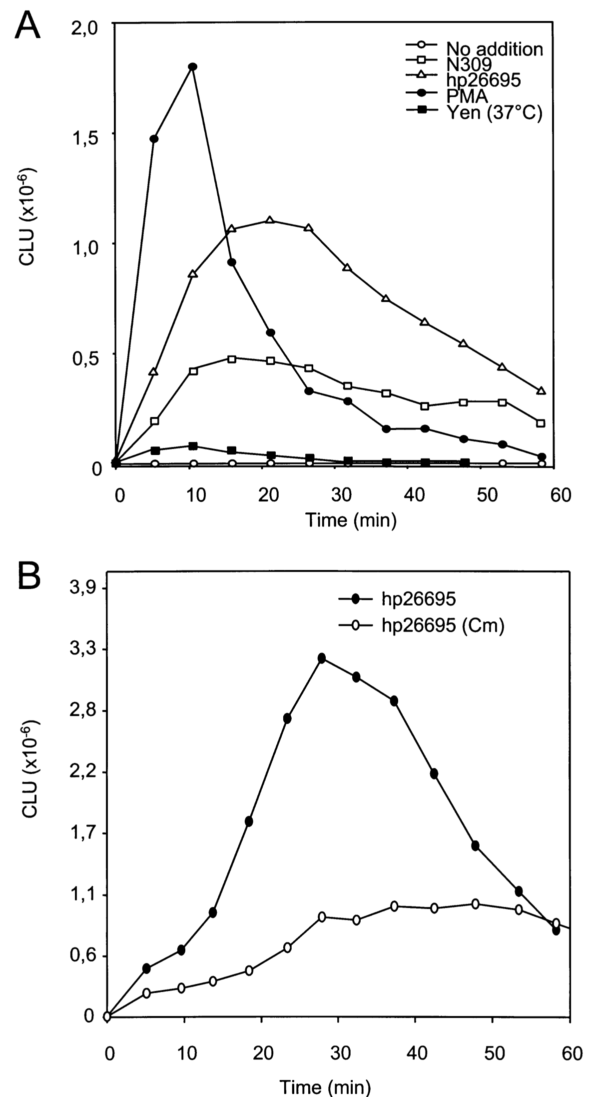

Fig. 1. Kinetics of oxidative bursts induced by *H. pylori and* other stimuli.
A. Oxidative burst response induced in PMNs (without additives, white circles) by hp26695 (white triangles), N309 (white squares), *Y. enterocolitica* (37°C) [Yen (37°C), black squares] or PMA (1 μg ml^{-1}; black circles). Bacteria were incubated at a multiplicity of infection (MOI) of 100 with freshly isolated PMNs, and the CL response was measured over 1 h. This is one of at least five different experiments showing similar results.
B. CL response in PMNs induced by hp26695 untreated (black circles) or treated with 38 μg ml^{-1} Cm for 2 h at 37°C (white circles). Bacteria were incubated at a MOI of 100 with freshly isolated PMNs, and the CL response was measured over 1 h. This shows one of at least five different experiments with similar results.

antiphagocytic properties (Lian *et al.*, 1987), did not induce any detectable oxidative response. It is pertinent to note that, in this and all subsequent experiments, *H. pylori* strains hp26695 and P76 gave similar results, and only one representative set of data is thus shown in each case throughout this study.

H. pylori*-induced oxidative burst depends on bacterial protein synthesis*

We found previously that exposing *H. pylori* to concentrations of chloramphenicol (Cm) that inhibit bacterial protein synthesis without killing the bacteria resulted in the opsonin-independent uptake of adherent *H. pylori* (Ramarao *et al.*, 2000). In order to determine what effect this treatment might have on the phagocyte's oxidative response, we treated *H. pylori* with 38 μg ml^{-1} Cm for 2 h at 37°C before infection. Cm treatment results in a 2.95 $\pm$ 1.11-fold reduction in CL response (Fig. 1B). This difference was not a result of lower numbers of bacteria in the Cm-treated samples, as bacterial densities in the culture supernatants were not significantly different during the 60 min infection (not shown). Furthermore, we have shown previously (Ramarao *et al.*, 2000) that Cm-treated and untreated bacteria adhere to phagocytic cells to the same extent. Together, this indicates that *de novo* protein synthesis is required to block *H. pylori* uptake, but enhances the oxidative response by phagocytes.

Opsonization of H. pylori *increases the phagocytic CL response*

The finding that Cm treatment of *H. pylori* results in a smaller CL response could mean that the phagocytic response is less intense when *H. pylori* is internalized. Although *H. pylori* that adhere to phagocytes in an opsonin-independent manner are not phagocytosed (Ramarao *et al.*, 2000), opsonization of these bacteria with human serum does lead to enhanced engulfment (Rautelin *et al.*, 1993; Ramarao *et al.*, 2000). In order to assess the phagocyte's response to *H. pylori* that have been internalized via opsonin-mediated processes, we incubated the bacteria with human serum before infection. This treatment resulted in a $>3.56 \pm 0.62$-fold increase in CL (Fig. 2). Parallel results have been seen previously with *Y. enterocolitica*, for which the phagocytosis and CL response were drastically increased in the presence of normal human serum (Lian and Pai, 1985). Yet, as increased uptake of Cm-treated *H. pylori* decreased the oxidative burst, these results indicate that, for *H. pylori*, the oxidative response is not directly related to the internalization process.

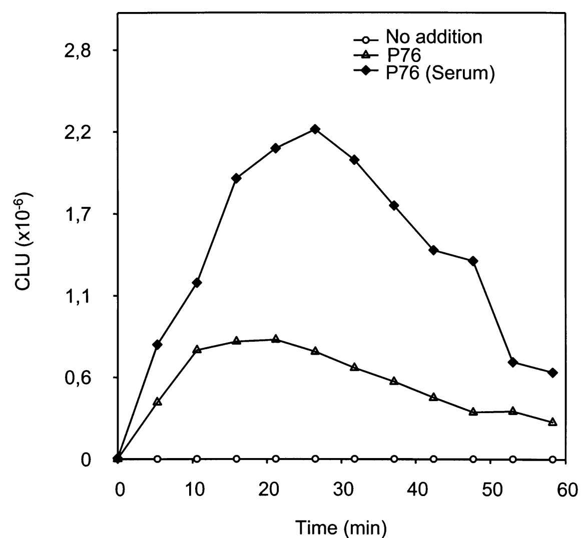

Fig. 2. CL response in PMNs (white circles) induced by P76 non-opsonized (white triangles) or opsonized with normal human serum for 30 min at 37°C (black diamonds). Bacteria were incubated at a MOI of 100 with freshly isolated PMNs, and the CL response was measured over 1 h. This is one of at least five different experiments with similar results.

H. pylori *survives the respiratory burst by professional phagocytes*

H. pylori blocks its own phagocytosis (Ramarao *et al.*, 2000), yet still induces a strong oxidative burst by both monocytes and PMNs (Fig. 1). We therefore aimed to determine whether the bacteria adherent to the phagocyte surface remained viable. Although dilution plating after host cell lysis has been used to quantify viable bacteria associated with infected cells, it does not allow us specifically to assess the viability of extracellular (i.e. versus intracellular) bacteria. As the coincident release of toxic phagocytic enzymes and metabolites cannot be avoided when such assays are used with phagocytes, these analyses are also of uncertain value when used in isolation.

In order to circumvent these problems, we tested the suitability of the Molecular Probes *Bac*Light fluorescence-based bacterial viability kit for our purposes. PMNs were infected for 1 h at 37°C with *H. pylori* in the presence of cytochalasin D (5 μg ml^{-1}), which completely blocks the phagocytosis of both live and dead *H. pylori* (data not shown). The infected cells were then treated with increasing concentrations of gentamicin (0–200 μg ml^{-1}) for 2 h at 37°C in order to kill increasing proportions of the bacteria. Replicate samples were either fixed with paraformaldehyde (PFA) and then stained using the *Bac*Light kit or serial dilutions of saponin lysates of the infected cells were plated. As illustrated in Fig. 3A, the two assays showed an excellent correlation between the proportion of bacteria that were viable at different gentamicin concentrations. As

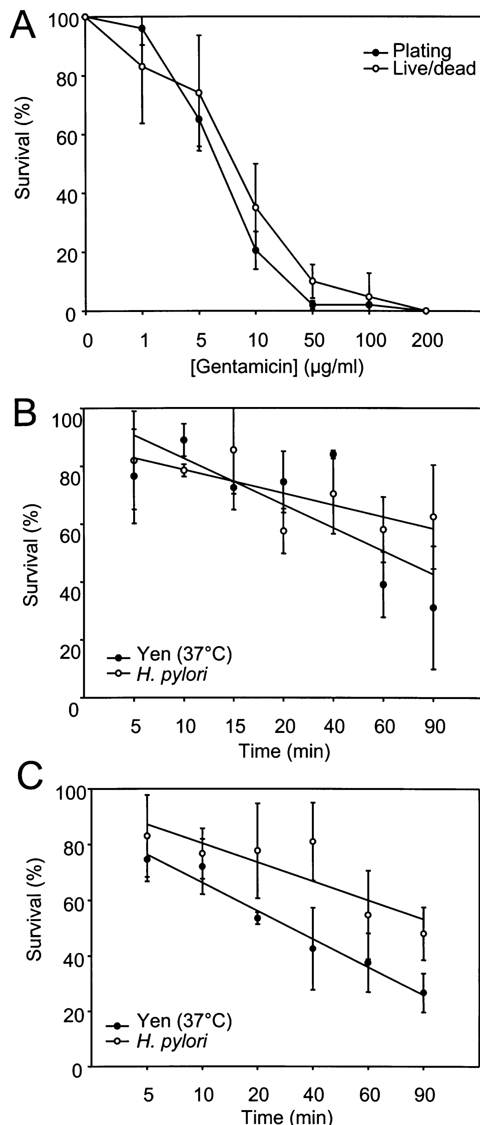

Fig. 3. Survival to the oxidative burst.
A. Comparison of the results obtained with live/dead kit (white circles) and plating (black circles) experiments. PMNs were incubated with cytochalasin D (5 μg ml^{-1}) and hp26695 for 1 h at 37°C. Afterwards, cells were treated with increasing concentrations of gentamicin (0–200 μg ml^{-1}) for 2 h at 37°C before being either stained using the live/dead kit or plated onto S plates. The values obtained for hp26695 not treated with gentamicin were considered as 100% survival in both experiments. These results were obtained from three different experiments, each done in triplicate.
B and C. Survival of hp26695 (white circles) and *Y. enterocolitica* (black circles) grown at 37°C on PMNs (B) and monocytes (C). Hp26695 and *Y. enterocolitica* were incubated with freshly isolated monocytes or PMNs at a MOI of 100 for up to 90 min at 37°C. At different time points, the infection was stopped by fixing the cells in PFA, and the infected cells were stained using the live/dead kit. The percentage of dead versus live bacteria was determined. This shows the results of four independent experiments, each done in duplicate.

it allowed us specifically to correlate bacterial localization with respect to the phagocyte, the *Bac*Light kit was used in all subsequent experiments.

When phagocytes were infected using our standard protocol (i.e. in the absence of cytochalasin D), most *Y. enterocolitica* remained viable (Fig. 3B and C), as was expected because this pathogen does not induce any detectable oxidative burst (Fig. 1). Despite the fact that *H. pylori* does induce a significant oxidative burst, the proportion of living bacteria associated with phagocytes was similar to that seen with *Y. enterocolitica*. Approximately 80% of *H. pylori* survived through the first 40 min of infection in both monocytes and PMNs. By 90 min, $>60\%$ of bacteria associated with PMNs and 50% of bacteria associated with monocytes still remained viable (Fig. 3B and C). As the peak CL response to *H. pylori* infection was typically achieved relatively quickly (see Fig. 1), the majority of adherent bacteria were able to resist this killing response.

Extracellular and intracellular oxidative burst

It is possible that *H. pylori* survival on the cell surface results from an ability to prevent the extracellular release of oxygen metabolites at the cell surface. For example, it has been demonstrated previously that non-opsonized *N. gonorrhoeae* induce a strong oxidative response by PMNs without causing the release of detectable amounts of reactive oxygen intermediates into the surrounding milieu (Naids and Rest, 1991). To determine whether *H. pylori* induced the release of any oxidative metabolites while persisting on the phagocyte surface, we dissected the luminol-dependent CL response into extracellular and intracellular events. Extracellular CL was measured by adding 1 mM NaN_3, which by itself totally abolishes the luminol-induced CL by inhibiting myeloperoxidase, and 4 U of horseradish peroxidase (HRP), an azide-insensitive peroxidase that regenerates a CL response in the presence of extracellular H_2O_2. Intracellular CL was measured by adding 2000 U of catalase, a high-molecular-weight H_2O_2 scavenger that abrogates extracellular CL by degrading H_2O_2 into O_2 and water (Naids and Rest, 1991). It is pertinent to note that sodium azide (SA), HRP and catalase did not themselves induce a CL response (data not shown; Suzuki *et al.*, 1992).

As controls to validate the system, the oxidative bursts induced by PMA, latex beads and *Y. enterocolitica* were assessed. As shown previously, the burst induced by PMA was almost exclusively extracellular (data not shown; Ewald *et al.*, 1994). In contrast, latex beads induced a response that was partially inhibited by both catalase and HRP/SA treatments, being 60% extracellular and 40% intracellular (Fig. 4A). Although *Y. enterocolitica* grown at 37°C totally inhibits the oxidative burst by both

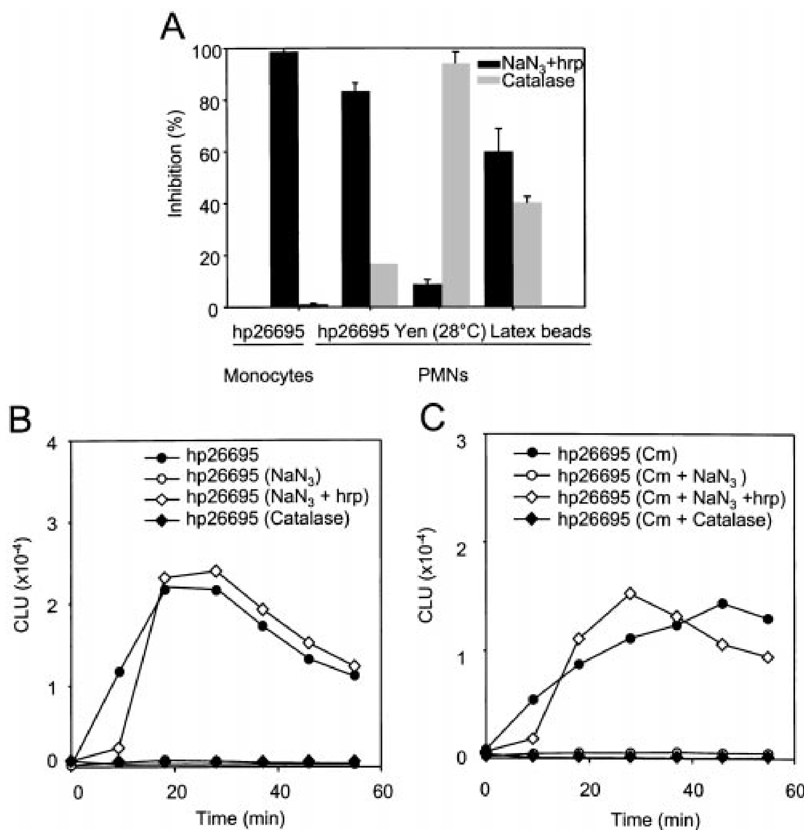

Fig. 4. Differentiation of extracellular and intracellular burst.
A. Extracellular and intracellular CL induced by hp26695 in PMNs and monocytes, and by latex beads and *Y. enterocolitica* grown at 28°C in PMNs. After addition of the bacteria or latex beads at a MOI of 100 bacteria or particles cell^{-1}, CL was measured continuously until peak CL response was reached. Results are expressed as standard deviation ($n = 4$) and represent the percentage CL extracellular or intracellular to the control neutrophils to which no additives were made. Extracellular CL was measured in the presence of 4 U HRP and 1 μg ml^{-1} sodium azide (NaN_3; black box), and intracellular CL was measured in the presence of 2000 U catalase (grey box) as described in *Experimental procedures*.
B and C. Extracellular and intracellular CL induced in monocytes by hp26695 untreated (B) and treated with 38 μg ml^{-1} Cm for 2 h (C; black circles). Intracellular CL was measured in the presence of 2000 U catalase (black diamonds), and extracellular CL was measured in the presence of 4 U HRP and 1 μg ml^{-1} NaN_3 (white diamonds) as described in *Experimental procedures*. NaN_3 alone completely inhibits the CL response (white circles). This shows one representative experiment out of four showing similar results.

monocytes and PMNs, when these bacteria are grown at 28°C instead, a burst does occur (data not shown; Ewald *et al*., 1994). In clear contrast to what was seen with either PMA or latex beads, *Y. enterocolitica* grown at 28°C induced a response that was almost exclusively intracellular (94%), as catalase did not prevent the measured CL response and HRP/SA did (Fig. 4A).

The CL responses of PMNs and monocytes to *H. pylori* infection were both inhibited by catalase, but not significantly affected by HRP/SA treatment. This clearly implies that oxygen metabolites are released into the extracellular milieu and, together with the results presented above, that *H. pylori* is able to survive exposure to these compounds.

One explanation for our results could be that the reactive oxygen intermediates are simply targeted to the associated bacteria. There is an extracellular burst in response to *H. pylori* because the bacteria are at the cell surface, whereas the phagocytosis of *Y. enterocolitica* grown at 28°C results in an intracellular burst. Alternatively, the different bacterial adhesin–host cell receptor interactions that mediate the adherence of these two pathogens to the phagocytes could result in different cellular responses. To test whether the internalization of viable *H. pylori* would lead to an intracellular burst, we treated these bacteria with sublethal concentrations of Cm to allow opsonin-independent uptake of the bound bacteria. As illustrated by comparing Fig. 4B and C, the oxidative response to treated versus untreated bacteria was almost exclusively extracellular. This implies that it is the mechanism of bacterial adherence to the phagocyte that results in the extracellular versus intracellular CL response.

H. pylori *survives the phagocyte's bactericidal activity, which is not completely inhibited by* H. pylori *infection*

Despite the fact that oxygen metabolites are released into the extracellular milieu, it is possible that the tight association between *H. pylori and* the phagocyte places the bacteria in a relatively innocuous microenvironment at the cell surface. In order to test whether this was the case, we performed co-infection experiments with *H. pylori and N. gonorrhoeae*. We have shown previously that *H. pylori* inhibits the opsonin-independent phagocytosis of *N. gonorrhoea*e (Ramarao *et al.*, 2000). We therefore assessed the viability of *N. gonorrhoeae* that remain tightly associated with the phagocyte surface after co-infection with *H. pylori.* In order to ensure that most gonococci remained extracellular, we first infected the phagocytes with *H. pylori* for 1 h before the addition of *N. gonorrhoeae.* After 1 h of co-infection, the samples were washed, fixed and then stained with the *Bac*Light kit as described above. Less than 25% of the *N. gonorrhoeae*

bound to the surface of PMNs survived the 1 h infection, whereas ≈ 60% of associated *H. pylori* survived 2 h of incubation with the phagocytes (i.e. 1 h before the addition of *N. gonorrhoeae* and then 1 h co-infection with *N. gonorrhoeae*; Fig. 5). The difference in survival was even more pronounced during the infection of monocytes, as ≈ 10% of gonococci versus 50% of *H. pylori* survived for the 1 h and 2 h infections respectively (Fig. 5). These results indicate that the infected phagocytes possess bactericidal activity and are able efficiently to kill cell surface-associated bacteria other than *H. pylori*.

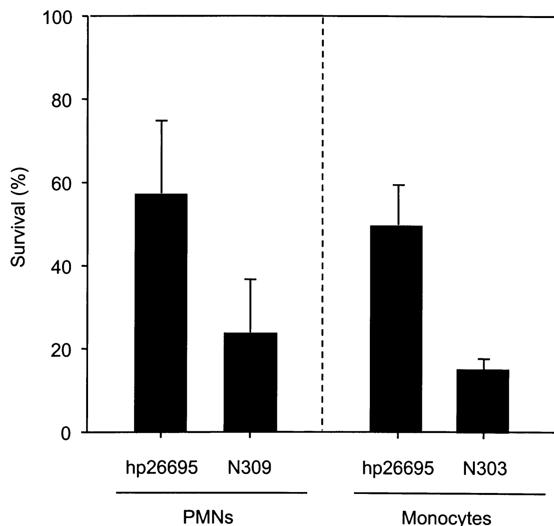

Fig. 5. Survival of adherent hp26695 and *N. gonorrhoeae* after co-infection of PMNs and monocytes. Hp26695 were incubated with freshly isolated PMNs or monocytes at a MOI of 100 for 1 h at 37°C before the addition of *N. gonorrhoeae* for 1 h. The infection was then stopped by fixing the cells in PFA, and the infected cells were stained using the live/dead kit. The percentage of dead versus live bacteria was determined. This shows the results of three different experiments, each done in duplicate.

Effect of catalase on the survival of H. pylori

As catalase almost completely inhibits the *H. pylori*-induced CL response (Fig. 4A and B), we therefore assessed the viability of attached *H. pylori* in the presence or absence of catalase. As seen in Fig. 6A, the viability of *H. pylori* at the surface of phagocytic cells was independent of the presence of catalase. Together with the comparison of the survival of *Y. enterocolitica*, these data imply a resistance mechanism in *H. pylori* to the toxic metabolites released by professional phagocytes. To analyse this resistance mechanism further, we tested the survival of a catalase mutant at the surface of the phagocytic cells. As seen in Fig. 6B, the survival of the catalase mutant decreases dramatically within 15 min after infection, compared with the wild type. However, the oxidative burst induced by this mutant was comparable with the one induced by the wild type (data not shown). Together, these data assign a crucial role to *H. pylori* catalase in the survival of this pathogen at the surface of these bactericidal cells.

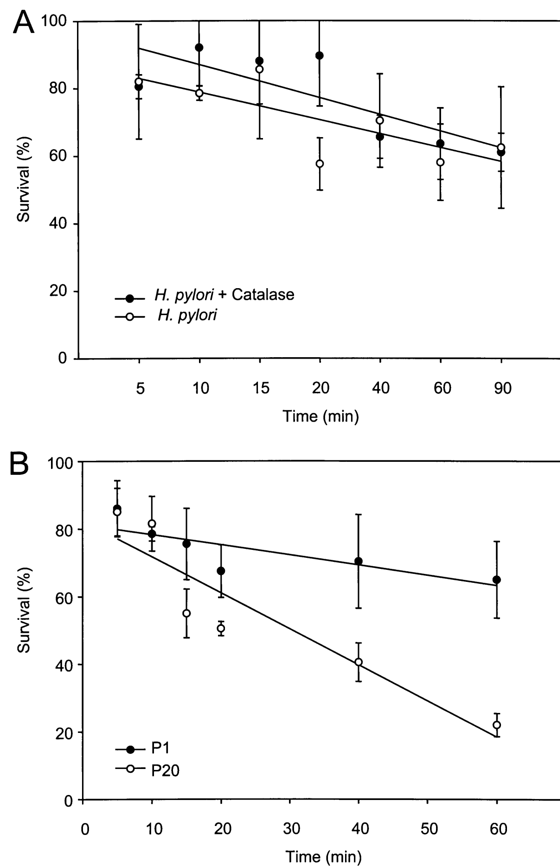

Fig. 6. A. Survival of *H. pylori* to the oxidative burst in the presence (black circles) or absence (white circles) of catalase.
B. Survival of *H. pylori* P1 (black circles) or of the catalase-deficient strain P20 (white circles). *H. pylori* hp26695, P1 or P20 were incubated with freshly isolated PMNs at a MOI of 100 for up to 90 min at 37°C in the presence or absence of 2000 U of catalase. At different time points, the infection was stopped by fixing the cells in PFA, and the infected cells were stained using the live/dead kit. The percentage of dead versus live bacteria was determined. This shows the results of four independent experiments, each done in duplicate.

Further characterization of the H. pylori-*induced oxidative burst*

The signal transduction cascades that trigger an oxidative response to many different stimuli have been well characterized (reviewed by Fantone and Ward, 1985). We therefore used a series of reagents to determine whether the signals that trigger the *H. pylori*-induced oxidative burst are different. The influence of extracellular Ca^{2+} on the phagocyte oxidative response has been

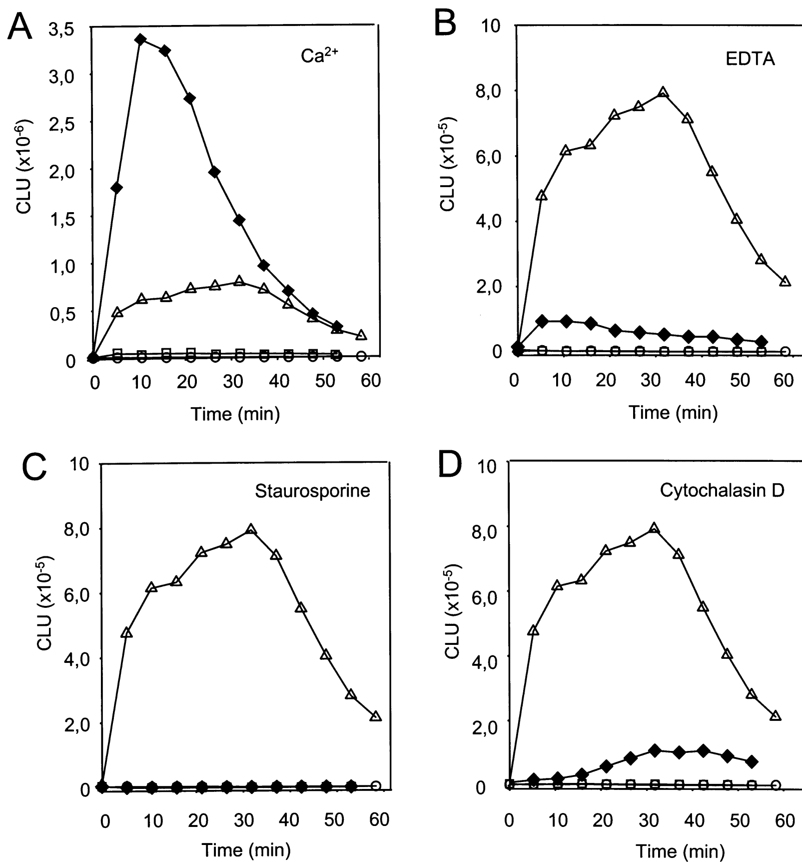

Fig. 7. CL responses obtained after the addition of several chemicals or inhibitors. Hp26695 was incubated with freshly isolated PMNs at a MOI of 100, and the CL response was measured over 1 h. The CL response was measured for PMNs alone (white circles), in the presence of hp26695 (white triangles) or with hp26695 and Ca^{2+} (A), EDTA (B), staurosporine (C) or cytochalasin D (D) (black diamonds), or for the tested chemical alone (white squares). This is a representative experiment of at least six different experiments with similar results.

described for various stimuli (Lew *et al.*, 1984). The addition of divalent cations to the CL reaction resulted in a sixfold increase in the CL burst induced by *H. pylori* (Fig. 7A), whereas the addition of EDTA, which chelates divalent cations including calcium, abrogated the induced response (Fig. 7B). As shown previously (Bass *et al.*, 1983), the addition of EDTA did not block the burst induced by PMA (Table 1), because this phorbol ester directly activates protein kinase C (PKC), an effect that normally occurs downstream of the induced transmembrane calcium flux. The addition of EDTA blocked the oxidative burst in response to latex beads, yet the addition of divalent cations had no effect on the amplitude of the response, implying that the trace amounts of Ca^{2+} present in standard CL reaction buffer was sufficient for a maximal response. As mentioned, the activation of PKC can induce an oxidative burst, and the protein kinase inhibitor staurosporine can block this response (Nielsen and Andersen, 1992). Consistent with this, staurosporine prevented any CL burst in response to PMA, latex beads or *H. pylori* (Fig. 7C; Table 1), implying a role for PKC in each of these processes. Cytochalasin D showed little effect on the oxidative burst induced by PMA, but completely inhibited that induced by latex beads and *H. pylori* (Fig. 7D, Table 1), implying that actin cytoskeletal rearrangements are required for the oxidative response to both of these particulate stimuli.

Table 1. Influence of various reagents on CL induced in PMNs and monocytes.

	Ca^{2+}	EDTA	Staurosporine	Cytochalasin D	Human serum
Latex beads	0	–	–	–	ND
PMA	0	0	–	0	ND
P76	+	–	–	–	+
Hp26695	+	–	–	–	+

Hp26695, P76, PMA or latex beads were incubated with freshly isolated PMNs or monocytes, and the CL response was measured over 1 h in the presence of Ca^{2+}, EDTA, cytochalasin D, staurosporine or normal human serum.
0, no effect on CL; +, increase in CL; –, blocking of CL; ND, not determined.

Discussion

Professional phagocytes, including polymorphonuclear (PMNs) and mononuclear cells (monocytes and macrophage), constitute a first line of host defence against confrontation with any invading microbe. It has been shown previously that *H. pylori* activates a chemotactic

response by both monocytes and PMNs, as evidenced by their increased adhesion to endothelial cells (Nielsen and Andersen, 1992; Rautelin *et al*., 1993; Yoshida *et al*., 1993). The bacteria's ability to survive despite the persistent inflammation that characterizes *H. pylori* colonization of the gastric mucosa implies that this important pathogen is able to evade local immune responses. We have demonstrated previously that *H. pylori* adheres to primary human neutrophils and monocytes in an opsonin-independent manner, but is not engulfed by these professional phagocytes (Ramarao *et al*., 2000). Sublethal doses of the bacterial protein synthesis inhibitor Cm resulted in *H. pylori* uptake, implying that *de novo* protein synthesis is required for the antiphagocytic function. Interestingly, the block in phagocytosis is not restricted to bound *H. pylori*, as *N. gonorrhoeae* and latex beads, both of which are normally engulfed efficiently, remained at the cell surface of phagocytes that had been infected before with *H. pylori* (Ramarao *et al*., 2000). This resistance to phagocytosis can be overcome by the presence of human serum, a fact that may help to explain the lack of bacteraemia associated even with persistent *H. pylori* infections.

In this study, we found that extracellular *H. pylori* remained viable (Fig. 3), despite the fact that a significant CL response was induced (Fig. 1). Other bacterial pathogens have evolved mechanisms to evade the oxidative response of phagocytes. For example, *Toxoplasma gondii* (Anderson and Remington, 1974), *Yersinia pestis* (Chanetzky *et al*., 1985), *Y. enterocolitica* (Lian *et al*., 1987), *Brucella abortus* (Kreutzer *et al*., 1979), *Salmonella typhi* (Kossack *et al*., 1981), *Chlamydia trachomatis* (Hammerschlag *et al*., 1985) and *Mycobacterium leprae* (Holzer *et al*., 1986) elicit little or no oxidative burst. In contrast, some adherent *N. gonorrhoeae* variants induce an enhanced oxidative burst (Knepper *et al*., 1997), but one that results in little release of oxidative metabolites into the extracellular milieu (Naids and Rest, 1991). The survival strategy of *H. pylori* appears to be different from any of the pathogens listed above. Almost all reactive oxygen metabolites generated are released into the extracellular milieu (Fig. 4), and the phagocyte's ability effectively to kill surface-associated *N. gonorrhoeae* (Fig. 5) implies that this is a noxious environment. Together, these data imply that *H. pylori* is not susceptible to the phagocytes' oxidative killing mechanisms. The reason for this resistance is uncertain; however, the fact that this bacteria has a very high catalase activity (Lee et al., 1993) and that the catalase mutant used in this study does not resist killing by PMNs (Fig. 6) makes it tempting to speculate that this peroxide-degrading property is an essential virulence factor.

Extracellular-associated and Cm-treated (i.e. phagocytosed) *H. pylori* both induce an almost exclusively extracellular CL response, whereas the uptake of other bacterial species (e.g. avirulent *Y. enterocolitica*) or particulate stimuli (e.g. latex beads) results in a response that is either partially or completely intracellular (see Fig. 4). We therefore tested the effects of several well-characterized reagents in order to determine whether the signal cascade that induces the oxidative burst after *H. pylori* binding resembled that described previously for other stimuli. Based upon this preliminary assessment, no obvious differences were seen compared with that which occurs downstream of the phagocytic uptake of latex beads (Fig. 6; Table 1). The fact that the *H. pylori*-induced burst leads to an extracellular release of oxygen metabolites and that it is inhibited in the presence of cytochalasin D resembles the phenomenon of frustrated phagocytosis described, for example, in the case of large beads (Liu *et al*., 1997). Further analyses of these processes are currently under way in order to understand why the targeted release of oxidative metabolites in response to these two particulate stimuli is different.

The fact that *H. pylori* induces an extracellular release of reactive oxygen metabolites has severe implications for the course of gastric diseases resulting from this pathogen. In addition to their effective bactericidal properties, such molecules are also toxic to host tissues. They could, for example, alter the viscoelastic properties of the mucous and cause direct damage to the mucosal epithelia. This could explain the inflammation-induced tissue damage and ulcer formation that is associated with persistent mucosal infection by *H. pylori*. It is interesting in this regard that certain clinical isolates have the ability to trigger an enhanced oxidative response by PMNs, and that their recovery appears to be associated with peptic ulcer disease and with a heavy inflammation in the gastric antrum and/or corpus, regardless of whether or not there is accompanying gastric ulcer (Rautelin *et al*., 1993). Such an association between an induced respiratory burst and increased clinical manifestations would seem to be paradoxical without the knowledge that this oxidative response is both ineffective in killing the bacterium and detrimental to adjacent host tissues.

Although it is pure speculation, the constant insult by reactive oxygen intermediates could also lead to DNA damage and may, therefore, contribute directly to the development of mucosal carcinomas that are associated with persistent *H. pylori* infection. Together, the finding that *H. pylori* can overcome the bactericidal activities of professional phagocytes has therefore opened an exciting new window into the pathogenic mechanisms of this pathogen that has only begun to be explored. Hopefully, these insights and those that result from them will allow new avenues to be explored towards the effective prevention and treatment of disease caused by this important pathogen.

Experimental procedures

Bacterial strains and growth conditions

H. pylori strain hp26695, kindly provided by D. Berg, St Louis, USA, is a type I strain originally isolated in the UK from a patient with gastritis; its complete genome sequence has been reported (http://www.tigr.org; accession number AE000511; Tomb *et al*., 1997). *H. pylori* strain P1 is a clinical isolate described elsewhere (Corthesy-Theulaz *et al*., 1996). *H. pylori* strain P76 is a spontaneous streptomycin-resistant strain derived from P49, a mouse-adapted strain originally provided by H. Kleanthous of OraVax, Cambridge, MA, USA (Gomez-Duarte *et al*., 1998). P20 is a *H. pylori* catalase mutant derived from P1 (Odenbreit *et al*., 1996), kindly provided by R. Haas (Munich, Germany).

H. pylori strains were grown on S plates [horse blood agar supplemented with, vancomycin (10 $\mu g\ l^{-1}$), nystatin (1 $\mu g\ l^{-1}$) and trimethoprim (5 $\mu g\ l^{-1}$)] 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.

N. gonorrhoeae strains N303 and N309 are pilus negative (Δ*pilE*) mutants that constitutively express the heparan sulphate receptor-specific Opa_{50} and CD66 receptor-specific Opa_{52} protein adhesins respectively (Kupsch *et al*., 1993; Gray-Owen *et al*., 1997b). Daily subculture of both *N. gonorrhoeae* strains to fresh GC agar was performed using a binocular microscope to select only those variants that expressed the single desired Opa protein.

The wild-type *Y. enterocolitica* serotype 0:9 strain used in these studies was kindly provided by J. Heesemann, Munich, Germany (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 proteins or at 28°C to allow bacterial phagocytosis and oxidative burst (Lee, 1997).

Isolation of PMNs and monocytes

Monocytes and PMNs were isolated using the methods described by Tomita *et al*. (1997). Briefly, the mononuclear and polymorphonuclear (PMNs) cell fractions were collected separately by standard Ficoll–Hypaque density gradient centrifugation (Amersham Pharmacia Biotech). Monocytes were separated from contaminating lymphocytes by incubating with glass coverslips at 37°C for 1 h. The attached monocytes were covered with RPMI-1640 medium before bacterial infection or resuspended in phosphate-buffered saline (PBS) for CL assays. Erythrocytes contaminating the PMN fraction were removed by hypotonic lysis, and PMNs were allowed to adhere to glass coverslips for 30 min. The attached PMNs were covered with RPMI-1640 medium before bacterial infection or resuspended in PBS for CL assays. 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 infection period.

Phagocytic infection experiments

Bacteria were resuspended from agar plates using cotton swabs, diluted in PBS 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. In co-infection experiments, cells were infected with *H. pylori* for 1 h before the addition of *N. gonorrhoeae*, and the infection was continued for 1 h. Infections were stopped at different time points by fixing the infected cells onto glass coverslips overnight at 4°C in PBS containing 3.7% PFA. After washing, the cells were stained using the live/dead staining kit as outlined below. All experiments were performed using both *H. pylori* hp26695 and P76 on monocytes and PMNs. In every case, the results were very similar, and only representative data for hp26695 are shown in this study unless otherwise indicated.

Bacterial viability assays

The live/dead *Bac*Light bacterial viability kit (Molecular Probes) was used to assess the viability of the bacteria at the surface of the phagocytic cells. This kit has been tested previously with a variety of organisms (Brunius, 1980; Davies, 1991), and our studies confirmed its utility with *H. pylori* (see below). After infection, cells were fixed onto the glass coverslips overnight at 4°C in 3.7% PFA. A mixture of the SYTO 9 green fluorescent nucleic acid stain and the red fluorescent nucleic acid stain propidium iodide was added to each well, and the samples were then incubated for 30 min in the dark. When used alone, the membrane-permeable SYTO 9 stain labels all bacteria in a population, whereas propidium iodide penetrates only those bacteria with damaged membranes. Because of a reduction in the SYTO 9 stain fluorescence when both dyes are present, bacteria with intact cell membranes will stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. After washing with PBS, coverslips were mounted in glycerol medium (Sigma ImmunoChemicals), sealed with nail polish and analysed using a Leica TCS 4D confocal laser scanning microscope (Leica Lasertechnik) equipped with an argon–krypton mixed gas laser. Images were taken serially using excitation and emission filters as appropriate for the fluorescent dyes employed. The excitation/emission maxima for these dyes are about 480–500 nm for SYTO 9 stain and 490–635 nm for propidium iodide. The proportion of live versus dead bacteria was calculated by counting the relative number of green versus red bacteria at the surface of infected cells. The phagocyte-induced killing was calculated by taking into account the proportion of viable bacteria in the absence of mammalian cells over the same time interval.

Measurement of phagocytic chemiluminescence (CL) response

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical)-enhanced CL was measured over 1 h at a constant temperature of 37°C using one of the two luminometers: Berthold models Lumat LB9501 or Multibiolumat LB9505C.

During the phagocytic respiratory burst, luminol acts as a bystander substrate for the oxidative species generated during activation of the phagocytic cells. The oxidation of luminol results in the production of an excited aminophthalate anion that relaxes to the ground state with the production of light (Allen *et al*., 1972; DeChatelet *et al*., 1982). Samples for CL were obtained by adding 0.2 ml containing 5×10^5 PMNs or monocytes in PBS to tubes containing luminol (20 μg ml^{-1}) and (i) 1 μg ml^{-1} PMA (Sigma Chemical); (ii) 5×10^7 latex beads (Difco Laboratories); or (iii) 20 μl of bacterial suspension containing 5×10^7 *H. pylori*, *N. gonorrhoeae* or *Y. enterocolitica*, as indicated. Where noted, the *H. pylori* suspensions were pretreated by incubating with 38 μg ml^{-1} chloramphenicol (Cm) for 2 h at 37°C or with human serum for 30 min at 37°C. In these cases, the bacteria were washed three times in PBSA before use in oxidative burst experiments.

We dissected the CL response into extracellular and intracellular events based upon the method of Naids and Rest (1991). Extracellular CL was measured by adding 1 mM sodium azide (NaN_3), which completely inhibits CL (extracellular and intracellular) by inhibiting the activity of myeloperoxidase, and 4 U of HRP, a membrane-impermeable azide-insensitive peroxidase that regenerates an extracellular CL response in the presence of H_2O_2. Intracellular CL was measured by adding 2000 U of catalase, a high-molecular-weight protein that abrogates extracellular CL by metabolizing H_2O_2 into water.

The role of different activators or inhibitors on oxidative burst was assessed by adding 0.5 mM Ca^{2+}, 10 mM EDTA, 5 μg ml^{-1} staurosporine or 5 μg ml^{-1} cytochalasin D, as indicated, to the samples immediately before measuring the CL response.

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