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Helicobacter pylori Lipopolysaccharide Hinders Polymorphonuclear Leucocyte Apoptosis

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SUMMARY: A prominent histologic feature of *Helicobacter pylori* infection is a dense infiltration of polymorphonuclear leukocytes (PMNL) in gastric mucosa. *H. pylori* lipopolysaccharide (LPS) has been recognized as a primary virulence factor evoking acute mucosal inflammatory reaction. Previous works have shown that *H. pylori* LPS immunologic activities are lower than those of enterobacterial LPS. However, the effect of *H. pylori* LPS on spontaneous PMNL apoptosis, and mechanisms by which this *H. pylori* LPS may promote PMNL survival remain to be established. In this study, we investigated, by both morphologic and biochemical approaches, the action of *H. pylori* LPS on PMNL apoptosis in vitro, using broth culture filtrates (BCF) of *H. pylori* strains with different genotypes. We found that BCF from *H. pylori* caused a significant delay in spontaneous PMNL apoptosis and this delay was independent of the VacA, *cag* pathogenicity island and urease status. We demonstrated that LPS in BCF is responsible for this effect because it was abrogated by the LPS antagonist B287 (a synthetic analog of *Rhodobacter sphaeroides* lipid A). Moreover, BCF from *H. pylori* induced P42/44$^{\text{MAP kinase}}$ activation in PMNL. Similar results were obtained with BCF of an *Escherichia coli* strain. Taken together these data suggest that longer survival of PMNL induced by *H. pylori* LPS may increase gastric epithelium injury in *H. pylori*–associated diseases. (*Lab Invest 2001, 81:375–384*).

Helicobacter pylori infection elicits an inflammatory cell response, and the severity of epithelial injury appears to be directly correlated with the extent of polymorphonuclear leukocyte (PMNL) infiltration (Davies et al, 1994; Mizuki et al, 2000). The severity of phagocyte recruitment is mainly associated with virulent type I strains of *H. pylori* that secrete a cytotoxin (VacA) and contain the *cag* pathogenicity island (PAI) (Blaser, 1997; Censini et al, 1996; Covacci et al, 1999; Labigne and de Reuse, 1996). Recruitment of PMNL is mediated by secretion of epithelial interleukin 8 (IL-8) induced by adherence of *H. pylori* at the apical side of epithelial cells (Crabtree et al, 1995; Hofman et al, 2000). It is thought that both bacterial components and host inflammatory mediators contribute to the subsequent tissue damage. More particularly, excess reactive oxygen metabolite production by PMNL has a pathogenic role in *H. pylori*–related gastroduodenal disease (Davies et al, 1994).

H. pylori is a noninvasive bacteria, but several bacterial products can enter the extracellular space and be in contact with PMNL in the lamina propria (Cao et al, 1998; Evans et al, 1995; Fiocca et al, 1999; Keenan et al, 1999; Mai et al, 1991, 1992; Schraw et al, 1999). The effects of bacterial products secreted by *H. pylori* or released after bacteria autolysis on the different functions of phagocytes (such as radical oxygen intermediates production, chemotaxis, phagocytosis, CD11b regulation) have been extensively studied (Akyon and Hascelik, 1999; Hansen et al, 1999a, 1999b; Harris et al, 1996; Mayo et al, 1997; Suzuki et al, 1992; Zhang et al, 1996). Among these products, lipopolysaccharides (LPS) may modulate the PMNL behavior. However, low immunologic activities of *H. pylori* LPS have been previously reported as compared with those of different bacterial LPS such as enterobacterial LPS (Muotiala et al, 1992; Nielsen et al, 1994). Interestingly, by inducing a low immunologic response, LPS may prolong *H. pylori* infection longer than would occur with a more aggressive pathogen (Moran, 1995). It has been suggested that the unusual structure of *H. pylori* lipid A might explain the reduced biologic activity of *H. pylori* LPS compared with other enterobacterial LPS (Geis et al, 1990; Moran, 1995).

Previous studies have demonstrated that PMNL activated with LPS from different enterobacteria or streptococci showed a longer survival compared with untreated PMNL (Colotta et al, 1992). Moreover, PMNL surviving in response to LPS retained their capacity to produce superoxide anion. In addition, prolongation of PMNL survival may be important for the regulation of host resistance and persistence of inflammation (Haslett, 1992; Savill et al, 1989). The effect of *H. pylori* LPS on PMNL survival has not yet been documented. The aim of the present study was to examine the relationship between PMNL survival in relation to LPS and to the genotypes for *cag* PAI, VacA, and urease.

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Results

Morphologic Study

Morphologic evaluation of PMNL cultured for 24 hours at 37° C revealed a statistically significant ($p < 0.01$) decrease in spontaneous PMNL apoptosis by the different broth culture filtrates (BCF) of *H. pylori*. By light microscopy, control PMNL showed a proportion of 5% ± 2% apoptotic cells after 2 hours of incubation (Fig. 1A) and 92% ± 8% apoptotic cells after 24 hours (Fig. 1B). PMNL incubated with BCF of different strains of *H. pylori* showed after 24 hours a reduced number of apoptotic cells (14% ± 5% and 16% ± 7% for 60190 strain and G21 strain, respectively) (Fig. 1, C and D). Using B287 (a synthetic analog of *Rhodobacter sphaeroides* lipid A), the proportion of apoptotic cells in PMNL treated with BCF from 60190 strain and its isogenic mutants (60190:v1 and 60190:C− strains) and in PMNL treated with BCF from G21 strain were similar to that of control cells after 24 hours: 85% ± 11% vs 90% ± 6% vs 82% ± 9% vs 89% ± 8% vs 90% ± 8% for 60190 strain (Fig. 1E), 60190:v1 strain (Fig. 1F), 60190:C− strain (Fig. 1G), G21 strain (Fig. 1H) and control cells, respectively. Apoptosis was induced similarly by staurosporine treatment in PMNL incubated with BCF from 60190 strain (Fig. 1I) and in control PMNL (Fig. 1J): 95% ± 5% vs 92% ± 8% for 60190 strain and control cells, respectively.

Evaluation of PMNL apoptosis by the TUNEL (TdT-mediated dUTP nick end labeling) method was performed at 12 hours of culture, because this method can detect apoptosis earlier than the Giemsa staining technique. However, using both methods, we obtained similar results; in control cells after a 2-hour culture period only rare apoptotic cells were detected (Fig. 2A), whereas most of control cells after a 12-hour culture revealed nuclear positivity (Fig. 2C). By contrast, only a few positive nuclei could be identified at 12 hours in PMNL treated with BCF from 60190 *H. Pylori* strain (Fig. 2B), G21 *H. pylori* strain (Fig. 2E), or *Escherichia coli* 055:B5 strain (Fig. 2F). B287 clearly antagonized the antiapoptotic effect of BCF from 60190 strain after 12 hours of culture (Fig. 2D). These results were also quantified by flow cytometry (means ± SEM, $n = 3$): 6% ± 2% and 81% ± 7% positive cells were observed after 2 hours and 12 hours of culture in the absence of BCF, respectively. BCF from 60190 *H. pylori* strain, G21 *H. pylori* strain, and *E. coli* 055:B5 strain, reduced the number of positive cells to 30% ± 5%, 28% ± 7%, and 32% ± 6%, respectively, after 12 hours of culture. Finally, B287 was found to block the antiapoptotic effect of BCF from 60190 *H. pylori* strain (84% ± 6% positive cells after 12 hours of culture).

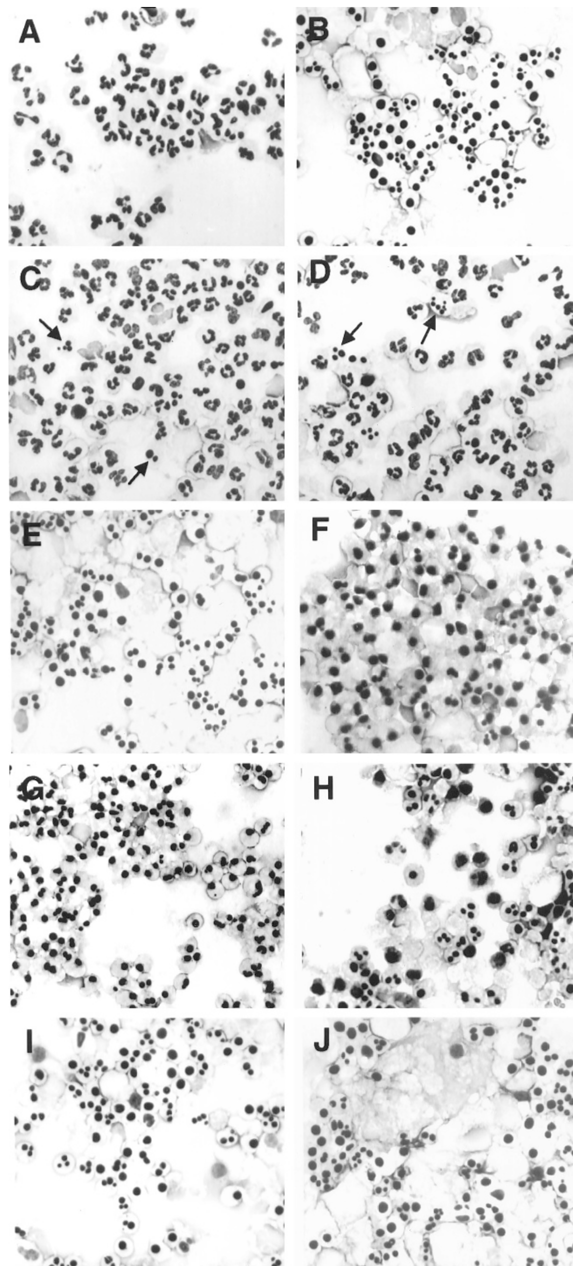

Figure 1.
Analysis of neutrophil apoptosis by morphologic evaluation of cultured PMNL. Control cells (2 hours of culture) show absence of apoptotic cells (A), whereas almost all cells at 24 hours of culture revealed nuclear condensation (B). Only a few apoptotic bodies and pyknotic nuclei (*arrows*) can be identified at 24 hours for the treated polymorphonuclear leukocytes (PMNL) with broth culture filtrate (BCF) from 60190 *Helicobacter pylori* strain (C) or with BCF from G21 *H. pylori* strain (D). Using B287, numerous apoptotic cells in PMNL treated with BCF from 60190 strain (E), in PMNL treated with BCF from G21 strain (F), in PMNL treated with BCF from 60190:v1 (G) and in PMNL treated with BCF from 60190:C− strain (H) were noted after 24 hours. All PMNL showed apoptotic features similar to staurosporine-treated cells with (I) or without (J) preincubation with BCF from 60190 *H. pylori* strain.

DNA Laddering

As shown in Figure 3, PMNL incubated with culture medium alone (RPMI 1640, 10% FCS) for 2 hours did not exhibit any increase in DNA laddering in comparison with control PMNL at 0 hours. Similar results were obtained with PMNL incubated for 2 hours with different BCF from *H. pylori* strains, with LPS from *E. coli* 055:B5, or with culture medium supplemented with 3 mM NH_4Cl. Staurosporine 1 μM, used as a positive control, strongly induced DNA fragmentation of PMNL. After 24 hours of culture, untreated PMNL

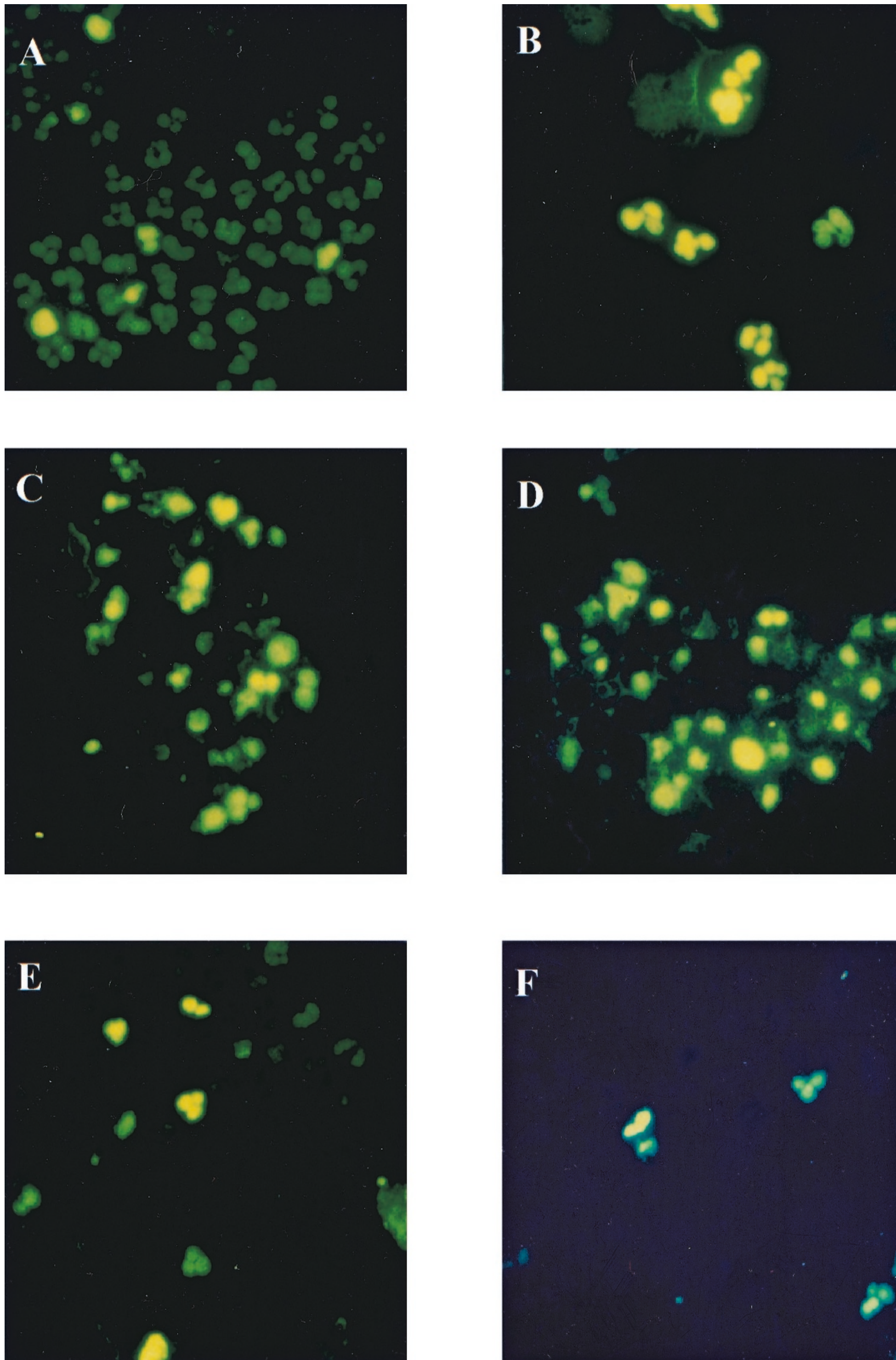

Figure 2.
Analysis of neutrophil apoptosis by TUNEL (TdT-mediated dUTP nick end labeling) method. A, Control cells at 2 hours of culture show a few apoptotic cells. B, Only a few positive nuclei can be identified at 12 hours in PMNL treated with BCF from 60190 *H. pylori* strain. C, Almost all control cells at 12 hours of culture revealed nuclear positivity. D, Using B287, numerous apoptotic cells in PMNL treated with BCF from 60190 strain were noted at 12 hours. E, Very few positive nuclei were observed at 12 hours in PMNL treated with BCF from G21 *H. pylori* strain and F, with BCF from *Escherichia coli*.

showed a significant increase in DNA fragmentation, whereas PMNL incubated for 24 hours with the different BCF of *H. pylori* strains (ie, 60190, G21, strain 4) or with LPS from *E. coli* 055:B5, exhibited a reduced amount of low molecular weight DNA in comparison with control PMNL (Fig. 3). Finally, BCF from 60190 strain has no effect on staurosporine-induced DNA fragmentation, whereas pretreatment of PMNL with B287 restored DNA degradation in the presence of BCF from 60190 strain (Fig. 3).

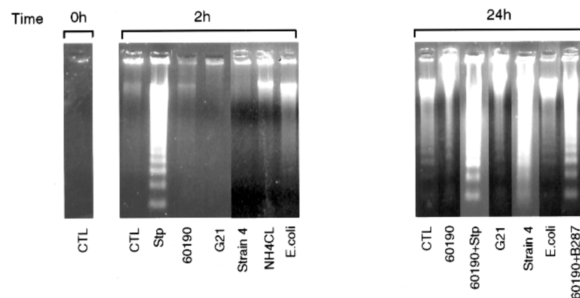

Figure 3.

DNA laddering analysis. DNA obtained, after 2 hours or 24 hours of culture, from control (*CTL*) PMNL and from treated PMNL, with either BCF from *60190*, *G21* and *Strain 4* strains of *H. pylori*, lipopolysaccharide (LPS) from *E. coli* 055:B5, NH_4Cl, staurosporine (*Stp*), or BCF from 60190 pretreated with *B287*, were separated on a 2% agarose gel containing ethidium bromide. The results are representative of three separate experiments using PMNL isolated from three different donors.

Procaspases 3 and 8 Activation in PMNL after 2- or 24-Hour Incubation with BCF from H. pylori *Strains*

We followed the activation of both caspases 3 and 8 by the cleavage of their respective zymogens of 32 and 52 kDa. As shown in Figure 4, staurosporine clearly induced the disappearance of procaspases 3 and 8, which reflects the activation of these two cysteine proteinase. BCF from various *H. pylori* strains clearly inhibited caspase 3 and caspase 8 activation at 2 hours and 24 hours. B287 significantly counteracted the effect of BCF from 60190 *H. pylori* strain at both 2 and 24 hours. As already reported for the TUNEL assay, BCF from the 60190 *H. pylori* strain failed to inhibit staurosporine-mediated activation of caspase 3 and caspase 8 (Fig. 4).

Measurement of Caspase Activity in PMNL Incubated with BCF from H. pylori

We next analyzed caspase activity in culture PMNL by using Ac-DEVD-pNA as substrate. Caspase activity was virtually undetectable in freshly isolated PMNL (Control 1), but was induced after 24 hours of culture (Control 2) (Fig. 5). Caspase activity in PMNL incubated 24 hours with BCF from 60190, 60190:v1, 60190:C−, 60190:M22, G21, and strain 4 *H. pylori* strains was strongly decreased compared with control PMNL cultured for 24 hours (1.5 ± 0.5 vs 1.2 ± 0.3 vs 1.4 ± 0.3 vs 1.1 ± 0.4 vs 1.4 ± 0. 2 vs 1.25 ± 0.5 vs 3.6 ± 0.3 mM/min·mg, for 60190 vs 60190:v1 vs 60190:C− vs 60190:M22 vs G21 vs strain 4 vs control, respectively, at 24 hours) (Fig. 5). Caspase activity in PMNL treated with NH_4Cl (3 mM) and in PMNL incubated with BCF from 60190 *H. pylori* treated with B287 was similar to control cells at 24 hours in culture (3.8 ± 0.4 vs 4 ± 0.3 versus 3.6 ± 0.3 mM/min·mg, for NH_4Cl-treated PMNL vs 60190 BCF treated with B287 vs control, respectively, at 24 hours). Similar results were obtained in PMNL incubated with BCF from 60190:v1 or 60190:C− or 60190:M22 *H. pylori*-treated with B287 (data not shown).

BCF from H. pylori *Induced P42/44^{MAPk} Activation in PMNL*

As shown in Figure 6A, P42/44^{MAPk} was rapidly induced in PMNL-treated with BCF. The effects of BCF from 60190 *H. pylori* strain was observed as soon as 2 minutes, was maximal at 5 minutes, and then rapidly decreased (Fig. 6A). The kinetics of BCF-mediated activation of P42/44^{MAPk} was indistinguishable from that of fMLP, which served as a positive control, although the extent of P42/44^{MAPk} phosphorylation was lower in BCF-treated cells than in fMLP-treated cells. No activation was noted in control PMNL. All BCF from *H. pylori* used in this study showed similar activation of P42/44^{MAPk} at 5 minutes, whereas this

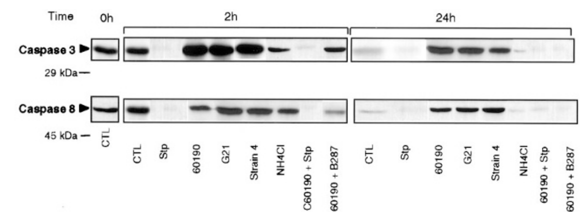

Figure 4.

Procaspase 3 and 8 activation in PMNL. After either 2 hours or 24 hours incubation with BCF from different *H. pylori* strains (60190, G21, strain 4), caspases 3 and 8 were strongly expressed in PMNL. Stp, staurosporine. The results are representative of three separate experiments using PMNL isolated from three different donors.

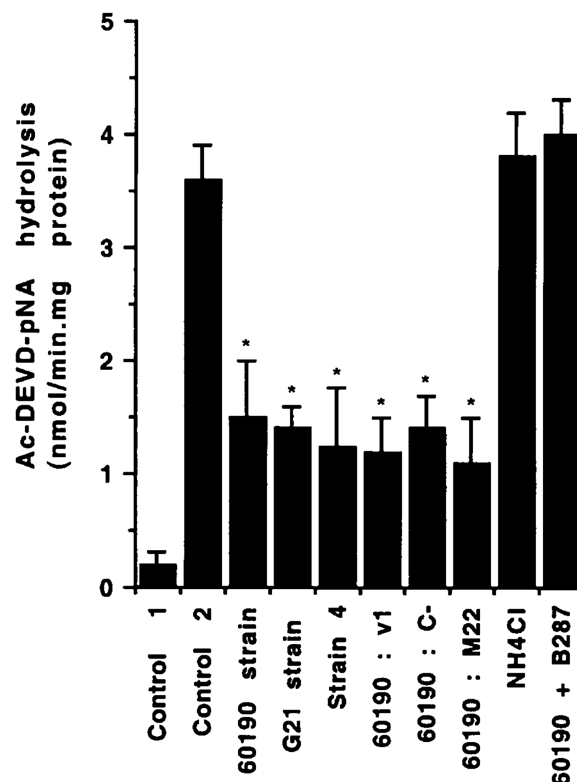

Figure 5.

Caspase 3 activity in PMNL measured with a continuous colorimetric assay. *Control 1*: caspase activity in PMNL at 0 hours in culture medium; *Control 2*: caspase activity in PMNL at 24 hours in culture medium. The results are the means of three independent experiments (*$p < 0.01$).

activation was strongly decreased by using BCF from 60190 treated with B287 or by using culture medium containing 3 mM NH_4Cl (Fig. 6A). PD98059 significantly attenuated BCF delay of PMNL apoptosis, because apoptosis was induced similarly by PD98059 treatment in PMNL incubated with BCF from 60190 strain and in control PMNL (Fig. 6B) (92% ± 5% vs 89% ± 8% for 60190 strain and control cells, respectively).

Discussion

The low biologic activity of *H. pylori* LPS has been extensively documented (Birkholz et al, 1993; Moran, 1995; Muotiala et al, 1992; Nielsen et al, 1994). In particular, *H. pylori* LPS induces a significantly lower oxidative metabolism, enzyme, and cytokine release than other known bacterial LPS (Birkholz et al, 1993; Nielsen et al, 1994). Previous studies have shown that there is a structure-function relationship of LPS and that lipid A is the biologically active part of the LPS molecule (Kanegasaki et al, 1986; Loppnow et al, 1989). The low potency of LPS from *H. pylori* is therefore probably due to the unusual acylation and phosphorylation pattern of its lipid A (Muotiala et al, 1992). In the present study, we provide for the first time evidence that, despite this low biologic potency, *H. pylori* LPS can dramatically hinder spontaneous PMNL apoptosis. This effect was similar to that obtained with LPS from *E. coli*.

Several *H. pylori* products can enter the extracellular space and interact with PMNL via several different mechanisms: (a) some proteins, like VacA, are specifically secreted (Schraw et al, 1999); (b) bacterial autolysis can release proteins (Phadnis et al, 1996); and (c) *H. pylori* releases membrane vesicles or blebs (Fiocca et al, 1999). Moreover, it has been demonstrated that LPS from several bacterial species can enter in the subepithelial space by epithelia transcytosis and have the capacity to stimulate basolaterally positioned PMNL to migrate into the paracellular space (Beatty and Sansonetti, 1997). VacA toxin is one of the major virulence factors produced by *H. pylori* (Cover, 1996), but its effect on PMNL behavior has not been well investigated. Urease on the *H. pylori* surface is a potent chemotactic factor for human leukocytes; it causes recruitment and activation of phagocytes, leading to gastric mucosal injury (Harris et al, 1996). Moreover, it has been reported that ammonia generated by *H. pylori* can reduce the production of reactive oxygen intermediates of PMNL (Mayo et al, 1997). Previous studies have demonstrated that ammonia evokes the depolymerization of PMNL actin cytoskeleton, decreases granule release induced by formylpeptides (Brunkhorst and Niederman, 1991), diminishes PMNL chemotaxis (Samanta et al, 1990), and inhibits fusion between phagosomes and lysosomes (Gordon et al, 1980). Finally, alkalinization of the environment due to release of ammonia may accelerate PMNL apoptosis (Leblebicioglu and Walters, 1999). The cag region of the *H. pylori* chromosome contains the gene encoding the cytotoxin-associated antigen (CagA) and may contain two adjacent genes, *picA* and *picB* (Blaser, 1997; Censini et al, 1996; Covacci et al, 1999). Gene products from cagA and picB may be present in the extracellular spaces and are implicated in the production, secretion, or anchorage of a protein involved in transducing the IL-8 inducing signal to epithelial cells (Cao et al, 1998; Crabtree et al, 1995). The effect of these products on PMNL behavior is still largely unknown. However, recent studies have shown that in PMNL, upregulation of CD11b, oxidative burst, and chemotaxis are not associated with the presence of *cag*A or *pic*B pattern (Akyon and Hascelik, 1999; Hansen et al, 1999a, 1999b). In our study, no relationship was found between hindered programmed cell death of PMNL in relation to genotypes for *cag* PAI, VacA, and urease: protection against apoptosis was not observed using BCF from the different strains tested, after removal of LPS with the antagonist B287. Finally our data highlight that LPS was the only component present in BCF obtained from different strains of *H. pylori* able to hinder PMNL apoptosis.

The hallmark of PMNL biology is their high capacity for spontaneous apoptosis. Rapid induction of apoptosis in PMNL, and the subsequent engulfment of the apoptotic cells by phagocytes are important in the rapid resolution of inflammation (Haslett, 1992; Savill et al, 1989). This is necessary to avoid unwanted tissue damage caused by the rapid release of the cellular contents of apoptotic PMNL (Haslett, 1992; Savill et al, 1989). It has been recently shown that caspases 3 and 8 are expressed in PMNL and that

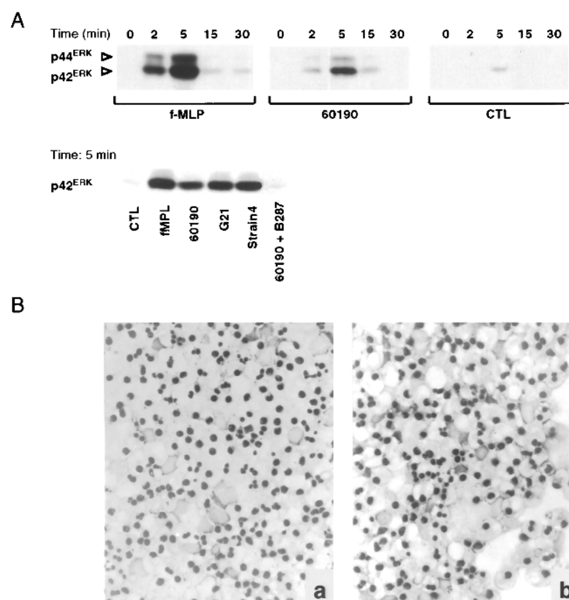

Figure 6.
A, P42/44^{MAPkinase} activity in PMNL at 5 minutes after incubation with BCF from different strains of *H. pylori* (60190, G21, strain 4), with NH_4Cl (3 mM), with staurosporine (1 μM). These P42/44^{MAPk} activations were compared with those obtained at 5 minutes for control (*CTL*) PMNL and for PMNL treated with formyl-Met-Leu-Phe (*fMLP*) (10^{-7} M) or with BCF from 60190 *H. pylori* strain. B, Effect of PD98059 treatment in PMNL incubated with BCF from 60190 strain of *H. pylori*: a, control cells at 24 hours, b, PMNL incubated with BCF from 60190 strain at 24 hours.

TNFα-induced PMNL apoptosis involves these caspases (Yamashita et al, 1999). In our study we showed that *H. pylori* LPS strongly inhibit procaspase 3 and 8 activation in PMNL at 24 hours. We next analyzed the involvement of $p42/44^{MAPk}$ in the protective effect of *H. pylori* LPS from BCF on PMNL apoptosis. Three distinct classes within the mitogen-activated protein (MAP) kinase family have been described, including $p42/44^{MAPk}$, JNK kinase, and p38, each having different physiologic roles (Paul et al, 1997). A specific function for MAP kinases has been proposed to play an important role in the respiratory burst, IL-8 production, and apoptosis of PMNL (Aoshiba et al, 1999; Frasch et al, 1998). Indeed, $p42/44^{MAPk}$ are involved in the protection of PMNL against apoptosis (Downey et al, 1998). In our study we demonstrated that LPS from *H. pylori* activates the $p42/44^{MAPk}$. This effect of *H. pylori* LPS on $p42/44^{MAPk}$ may account for the inhibitory effect of BCF from *H. pylori* on PMNL apoptosis. Moreover, PMNL undergo marked phenotypic changes during the process of spontaneous apoptosis, such as reduced expression of CD62L, CD15, CD16, and CD43 and increased expression of CD11b/CD18 and CD11c/CD18 (Dransfield et al, 1995; Homburg et al, 1995). Finally, in addition to LPS, it has been shown that PMNL apoptosis is delayed by several cytokines, such as granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, IL-8, and IFNγ (Colotta et al, 1992; Klein et al, 2000; Lee et al, 1993, Leuenroth et al, 1998; Yamamoto et al, 1993).

The exact mechanisms involved in the protective effect of LPS on programmed cell death in human PMNL are presently not well understood. However inhibition of PMNL apoptosis by LPS greatly prolonged their functional longevity as assessed by a number of parameters, including chemotaxis, phagocytosis, and stimulated secretion (Lee et al, 1993). In vivo, *H. pylori* LPS is probably not the only mechanism that can prevent or hinder the apoptosis of PMNL. Proinflammatory cytokines, such as IL-8 or GM-CSF, can be released by epithelial cells after bacterial infection, and these cytokines can prevent PMNL apoptosis (Hofman et al, 2000; Klein et al, 2000; Leuenroth et al, 1998; Yamamoto et al, 1993). However, considering that only some virulent strains of *H. pylori* are able to induce IL-8 secretion by epithelial cells (Hofman et al, 2000), our results show that *H. pylori* strains can hinder PMNL apoptosis independently of the cag status. Although *H. pylori* LPS has not yet been demonstrated in the lamina propria, bacterial LPS may be in contact with PMNL after epithelium destruction or tight junction opening, or after transepithelial migration. Finally, as shown for *Shigella* LPS, *H. pylori* LPS may be released in the subepithelial space and may be in contact with PMNL (Beatty et al, 1999).

The consequences of the increased survival of PMNL in the gastric mucosa induced by *H. pylori* LPS may be to induce gastric epithelium injury by excessive release of proteinases and radical oxygen intermediates. Thus, inhibition of apoptosis during infection may contribute greatly to the severity of epithelium injury.

In conclusion, although the immunogenicity and biologic activity of the *H. pylori* LPS on PMNL is low, it nevertheless appears to dramatically contribute to pathogenicity by modulating PMNL programmed cell death. This delay in PMNL apoptosis may enhance inflammation and result in aggravation of PMNL-mediated tissue damage in *H. pylori*-associated diseases. Elucidation of the precise roles in vivo of this delay may provide therapeutic advantages against *H. pylori* infection.

Materials and Methods

Bacterial Strains and Preparation of BCF

We used the urease^{+}/VacA^{+}/*cag*$^{+}$ wild-type *H. pylori* 60190 strain (American Type Culture Collection 49503; Rockville, Maryland), its urease-negative spontaneous mutant (strain 4) (Perez-Perez et al, 1992), and its isogenic mutants in which *vac A* (60190:v1) or *cagA* (60190:M22) or *picB*/*cagE* (60190:C−) genes were disrupted by insertional mutagenesis (Cover et al, 1994; Ghiara et al, 1995; Tummuru et al, 1994, 1995) (these strains have been kindly given by T. L. Cover and M. J. Blaser, Nashville, Tennessee). In addition, we used the wild-type G21 (urease^{+}/VacA^{-}/*cag*$^{-}$) *H. pylori* strain (kindly given by N. Figura, Sienna, Italy) (Ghiara et al, 1995). BCF were prepared as previously described (Ricci et al, 1997). Briefly, bacteria were grown in *Brucella* broth (Difco, Detroit, Michigan) supplemented with 1% Vitox (Oxoid, Basingstoke, United Kingdom) and 5% FCS (GIBCO BRL, Paisley, Scotland) for 24 to 36 hours at 37° C in a thermostatic shaker under microaerophilic conditions. When the bacterial suspensions reached 1.2 units of optical density at 450 nm (corresponding to a bacterial concentration of 5×10^8 CFU/ml), bacteria were removed by centrifugation, and the supernatants were sterilized by passage through a 0.22-μm pore-size cellulose acetate filter to obtain BCF. BCF were diluted 1:3. The vacuolating power of VacA^{+} BCF prepared and used as described is equivalent to that exhibited by a final concentration of 0.4 μg/ml purified VacA (VR, personal observation). For some experiments we also used BCF from *E. coli* 055:B5 strain. The concentrations of LPS in BCF used in this study were evaluated with a limulus test (BioWhittaker, Walkersville, Maryland).

PMNL Preparation and Incubation

Human PMNL were isolated from whole blood using a gelatin-sedimentation technique (Hofman et al, 1996). Briefly, whole blood anticoagulated with citrate/dextrose was centrifuged at 300 $\times g$ for 20 minutes (20° C). The plasma and buffy coat were removed and the gelatin/cell mixture was incubated at 37° C for 30 minutes to remove contaminating red blood cells. Residual red blood cells were then lysed with isotonic ammonium chordee. After washing in HBSS without Ca^{2+} or Mg^{2+}, the cells were counted and resus-

pended at 5×10^7 PMNL/ml. PMNL (95% pure) with 98% viability by trypan blue exclusion were used within 1 hour after isolation.

PMNL were incubated with *H. pylori* BCF containing 400 ng/ml LPS. In some experiments *E. coli* LPS (*E. coli* 055:B5 LPS; Sigma, Paris, France) (400 ng/ml) was used as a control. In other experiments the LPS antagonist B287 (Eisai Institute, Andover, Massachusetts) was added to the different BCF before incubation with PMNL (Golenbock et al, 1991). B287 is a synthetic compound whose structure is identical to the proposed structure of *R. sphaeroides* lipid A (Golenbock et al, 1991).

Morphologic Analysis

Morphologic changes of apoptosis were demonstrated by light microscopic examination of Wright-Giemsa-stained cytospins. Briefly, control PMNL and treated PMNL were fixed with methanol, stained, and the slides were examined by oil-immersion light microscopy (original magnification, ×1,000). At least 400 cells of each preparation in different fields of view were counted. Apoptotic cells were easily distinguishable by their reduced volume, chromatin condensation, and nuclear fragmentation.

Detection of apoptotic PMNL was performed by using TUNEL staining. TUNEL staining of the apoptotic cells was performed as described previously (Karahashi and Amano, 1998). In brief, PMNL were seeded at 4×10^4 cells/100 μl of the medium into the wells in a slide glass, and preincubated. The cells were fixed directly with 3% formaldehyde in the culture medium, pH 7.0, extensively washed with PBS, and stained with an Apoptosis in Situ Detection Kit (Roche, Paris, France) according to the manufacturer's protocol. Stained cells were observed using a laser scanning fluorescence microscope (Leica, Lyon, France) equipped for epifluorescence, and photographs were taken in random fields. To better quantify the apoptotic cells, 1×10^6 PMNL per condition were fixed in paraformaldehyde 3.7% and stained using the TUNEL assay as described above, and were analyzed by flow cytometry using a FACS Becton Dickinson (Paris, France).

DNA Fragmentation Assay

DNA was isolated from 1×10^7 control and treated PMNL. PMNL DNA fragmentation was conducted according to the procedure for assaying DNA fragmentation in total genomic DNA. In brief, the cells were lysed with TES buffer (20 mM Tris HCL, 200 mM EDTA, and 1% SDS) with RNAse (20 μg/ml; Boehringer Mannheim, Indianapolis, Indiana) at 37° C for 1 hour. Proteins were denatured by incubation with proteinase K (1 mg/ml; Boehringer Mannheim) at 55° C for 3 hours. The denatured protein was removed by phenol extraction. The DNA was then precipitated with alcohol overnight at −20° C. DNA was rinsed with alcohol, mixed with loading buffer, and then electrophoresed in a 2% agarose gel containing 10 μg/ml ethidium bromide. The gel was examined and photographed under ultraviolet light to detect a regular DNA fragmentation pattern (laddering) characteristic of apoptosis.

Western Blotting for Caspases 3 and 8 and $p42/44^{MAPk}$

Control and treated PMNL were washed in Hanks' balanced salt solution (Sigma), then in lysis buffer at 4° C (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 2 mM EDTA, 1% NP40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 25 μM leupeptin, 5 mM benzamidin, 1 μM pepstatin, 25 μM aprotinin, 50 mM sodium β-glycerophosphate, 20 mM sodium pyrophosphate, 0.5 mM dithiothreitol) at a density of 5×10^7 cell/ml. After sonication (2 pulses of 8 seconds each), lysates were centrifuged at 15,000 $\times g$ for 15 minutes at 4° C and denatured by boiling in reducing SDS sample buffer. Protein lysates (50 μg per sample) were analyzed by migration in SDS-PAGE and subsequently were electrophoretically transferred to nitrocellulose membrane. Nitrocellulose membrane was incubated in saturation buffer then probed with anti-phospho-$p42/44^{MAPk}$ (diluted 1:2,000) (New England BioLabs, Boston, Massachusetts) antibody incubated overnight at 4° C. This labeling was visualized by peroxidase-conjugated secondary antibodies (antirabbit IgG) (1:3,000) (Dakopatts, Copenhagen, Denmark) and by enhanced chemiluminescence (ECL kit; Amersham International, Buckinghamshire, United Kingdom). As positive control for $p42/44^{MAPk}$ activation, we used the chemoattractant formyl-Met-Leu-Phe (fMLP) (10^{-7} M), because it was previously demonstrated that fMLP both activated $p42/44^{MAPk}$ and delayed PMNL apoptosis (Avdi et al, 1996; Murray et al, 1997). To determine if *H. pylori*-LPS delays PMNL apoptosis by activation of $p42/44^{MAPk}$, we used the ERK pathway inhibitor PD98059 (Biomol Research Laboratories, Plymouth, Pennsylvania), 1 hour at 10 μM, before PMNL incubation with the different BCF from *H. pylori* as described above. PMNL apoptosis was evaluated by light microscopy as previously described in this study.

Ac-DEVD-pNA Cleavage Assay

Caspase activity was measured using kinetic colorimetric assay. Briefly, control or treated PMNL were sonicated (2 pulses of 8 seconds each), and then the lysates were centrifuged at 15,000 $\times g$. Cell extracts (50 μg per sample) were incubated with 200 μM of Ac-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA; Alexis Corporation, Paris, France) preferentially cleaved by members of the caspase 3 family of cysteine protease. Liberation of pNA was monitored continuously at 37° C by using an excitation wavelength of 410 nm. Measurements were recorded over the linear range of the assay, and caspase activity was controlled by adding to the cell extract an apopain/CPP-32 inhibitor (Ac-DEVD-CHO; Alexis Corporation). Substrates without lysates served as negative controls.

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