The Neutrophil-activating Protein (HP-NAP) of *Helicobacter pylori* Is a Protective Antigen and a Major Virulence Factor

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Abstract

Helicobacter pylori infection induces the appearance of inflammatory infiltrates, consisting mainly of neutrophils and monocytes, in the human gastric mucosa. A bacterial protein with neutrophil activating activity (HP-NAP) has been previously identified, but its role in infection and immune response is still largely unknown. Here, we show that vaccination of mice with HP-NAP induces protection against *H. pylori* challenge, and that the majority of infected patients produce antibodies specific for HP-NAP, suggesting an important role of this factor in immunity. We also show that HP-NAP is chemotactic for human leukocytes and that it activates their NADPH oxidase to produce reactive oxygen intermediates, as demonstrated by the translocation of its cytosolic subunits to the plasma membrane, and by the lack of activity on chronic granulomatous disease leukocytes. This stimulating effect is strongly potentiated by tumor necrosis factor α and interferon γ and is mediated by a rapid increase of the cytosolic calcium concentration. The activation of leukocytes induced by HP-NAP is completely inhibited by pertussis toxin, wortmannin, and PP1. On the basis of these results, we conclude that HP-NAP is a virulence factor important for the *H. pylori* pathogenic effects at the site of infection and a candidate antigen for vaccine development.

Key words: neutrophils • monocytes • NADPH oxidase • chemotaxis • *Helicobacter*

Introduction

Infection of the stomach mucosa by *Helicobacter pylori* is accompanied by a large infiltration of the mucosa by neutrophils, which are believed to contribute substantially to *H. pylori*–induced gastritis (1–5). Indeed, there is a good correlation between the degree of mucosal damage and neutrophil infiltration (1, 6, 7). Several studies have provided evidence for the presence of protein component(s) in *H. pylori* water extracts that attract and activate neutrophils and other inflammatory cells (8–17). Moreover, *H. pylori* strains capable of neutrophil activation were found more frequently in patients affected by peptic ulcer disease than active chronic gastritis only (18).

An *H. pylori* protein capable of promoting neutrophil adhesion to endothelial cells was purified and found to be a 150-kD decamer composed of identical subunits (19, 20). It was termed *H. pylori* neutrophil-activating protein (HP-NAP)[1] because it induced neutrophils to adhere to endothelial cells and to reduce nitroblue tetrazolium (NBT), via the production of reactive oxygen intermediates (ROI) (20). A considerable variation in the level of neutrophil adhesion promoting activity among different *H. pylori* strains was documented (20), suggesting a variable

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[1]*Abbreviations used in this paper:* [Ca^{2+}]$_i$, intracellular Ca^{2+} concentration; CGD, chronic granulomatous disease; DPI, diphenyleneiodonium chloride; HP-NAP, *Helicobacter pylori* neutrophil-activating protein; HRP, horseradish peroxidase; NBT, nitroblue tetrazolium; PI3-K, phosphatidylinositol 3-kinase; PLC, phospholipase C; ROI, reactive oxygen intermediates.

level of expression of the protein, similar to what was found for VacA (21). HP-NAP is localized in the bacterial cytosol and is released upon autolysis. HP-NAP can bind to the external surface of the outer membrane (22), in a similar manner as urease (23). In such a location, HP-NAP can mediate the binding of *H. pylori* to the cell surface via interaction with carbohydrates (24). Thus, notwithstanding the possible role of HP-NAP in *H. pylori*–associated diseases and in the human immune response to the bacterium, very little is known about these aspects of HP-NAP activity.

Very recently, computer-assisted molecular modelling, and spectroscopic and electron microscopic analysis indicated that HP-NAP is a 4-helix bundle protein forming dodecamers capable of binding a maximum of 500 atoms of iron per oligomer (25). Database searches indicate that HP-NAP–like molecules are present in a variety of pathogenic bacteria, but no data on their biological activities are reported. Thus, HP-NAP appears to be the prototype for a highly conserved class of bacterial homooligomeric proteins, and further studies are needed to characterize their activity particularly with respect to the immune and inflammatory responses.

Here, we show that HP-NAP is important both for immunity and for pathogenesis. Indeed, we found that the majority of the infected patients have antibodies against this antigen, and that vaccination of mice with HP-NAP induces protection against a subsequent challenge with *H. pylori*. Furthermore, we show that HP-NAP is chemotactic for neutrophils and monocytes and that it induces ROI production in humans by activating the plasma membrane NADPH oxidase via a signaling pathway involving trimeric G protein, phosphatidylinositol 3-kinase (PI3-K), Src family tyrosine kinases, and rise in cytosolic calcium. TNF-α and IFN-γ prime neutrophils in such a way as to potentiate the effect of HP-NAP. These results identify HP-NAP as a virulence factor relevant for the pathogenic effects of *H. pylori* at the sites of infection, and point to HP-NAP as a possible candidate for a multicomponent vaccine against *H. pylori*.

Materials and Methods

Reagents. HP-NAP was cloned and expressed in *Bacillus subtilis* to avoid contamination with LPS. Two preparations of HP-NAP were isolated from *H. pylori* CCUG strain as described previously (20). CagA and *H. pylori* extracts were prepared as previously (16, 26, 27). The purification and the characteristics of the LTK63 mutant of *Escherichia coli* heat-labile enterotoxin have also been reported (28). TNF-α and IFN-γ were obtained from Società Italiana Chimici; FMLP, wortmannin, homovanillic acid, horseradish peroxidase (HRP), and Fura-2 were purchased from Sigma Chemical Co.; diphenyleneiodonium chloride (DPI) and PP1 were from Calbiochem; pertussis toxin was prepared as described (29). Anti-p47[phox], anti-p67[phox], and anti-p40[phox] affinity-purified rabbit polyclonal antibodies were a gift of Dr. F. Wientjes (Department of Medicine, University College, London, UK). Solutions used throughout the experiments were prepared with endotoxin-free water for clinical use.

Assay of HP-NAP–specific Antibodies and Mice Immunization. Serum samples from 35 healthy adults, known to be positive for *H. pylori*, were tested for the presence of anti–HP-NAP, anti-CagA, and anti–*H. pylori* IgG antibodies using an ELISA originally reported by Xiang et al. (30) with minor modifications. Some of these serum samples were also tested by immunoblotting, using an *H. pylori* sonicate, as described (30).

CD-1–specific pathogen-free mice were immunized orally and then challenged with *H. pylori* according to described procedures (16, 26). In brief, groups of 10 mice (Charles River) were immunized intragastrically at days 0, 7, and 14 with saline alone (control) or with saline containing 100 mg of *H. pylori* CagA, glutathione *S*-transferase (GST)–HP-NAP, or *H. pylori* lysate together with 10 mg of LTK63 mutant as a mucosal adjuvant. At days 21, 23, and 25, all mice were challenged intragastrically with 10^9 CFU of *H. pylori* strain SPM326, a clinical isolate that has been adapted to colonize the mouse, as described previously in detail (16). At day 35, mice were killed, the stomachs were removed, and colonization was determined by culture as reported (26). Mice were considered as protected (not infected) when no *H. pylori* colony was detected on the stomach culture plates.

Cells. Neutrophils and monocytes (95–98% purity) were isolated from buffy coats of healthy donors or patients with chronic granulomatous (CGD), as reported previously (31, 32).

Assays of Chemotaxis and $\beta2$ Integrin Expression. Neutrophil migration was assessed using 3-μm-pore transwells (growth area 1 cm^2; Costar). Neutrophils were suspended in PBS containing 1 mM CaCl$_2$, 1 mM MgCl$_2$, and 10% FCS, pH 7.2, at 2×10^6/ml, and 300 μl of the cell suspensions was added to the top well while 1.2 ml medium containing the agonist was added to the bottom well. After fixation with 1.5% glutaraldehyde, migrated cells were counted by FACS® using polystyrene beads (Polysciences) as an internal standard (33).

Expression of $\beta2$ integrins was evaluated by FACS® analysis with the mouse anti–human CD18 mAb IB4. In brief, neutrophils and monocytes were incubated at 37°C for 30 min in the presence of HP-NAP or FMLP, centrifuged, and then stained with an FITC-conjugated rabbit anti–mouse polyclonal antibody. After extensive washing, cells were analyzed with a Becton Dickinson FACS® apparatus.

Stimulation of ROI Production. The production of ROI was measured in stirred cell suspension (10^6/ml) in HBSS at 37°C as H$_2$O$_2$-HRP–dependent oxidation of homovanillic acid by using a spectrofluorimeter (model LS 50B; PE Biosystems) as described previously (34).

Measurement of Cytosolic Ca^{2+}. Ca^{2+} was monitored in neutrophils loaded with 2 μM Fura-2 (32, 34, 35) for 25 min at 37°C. After loading, cells were diluted fivefold with HBSS, and incubation was prolonged for 25 min at 37°C. The cell suspension was centrifuged at 1,000 rpm for 5 min, and the pellet was resuspended in HBSS at a final concentration of 2×10^7 PMNs/ml. 10^6 cells/ml were treated with HP-NAP or FMLP (1 μM final concentration), and changes in cytosolic calcium concentration were measured by fluorescence spectroscopy in an LS 50B apparatus (excitation wavelength 335–380 nm, and emission 509 nm; PE Biosystems). Discrimination between calcium release from intracellular stores and extracellular influx was achieved by addition of 2 mM EGTA for 60 s at 37°C before agonist treatment.

Inhibitory Effect of Pertussis Toxin, Wortmannin, DPI, PP1, TNF-α, and IFN-γ. To evaluate the effect on the respiratory burst, 10^6 cells/ml were treated with 800 ng/ml of pertussis toxin for 120 min at 37°C; wortmannin for 5 min, or DPI (10 μM) or PP1
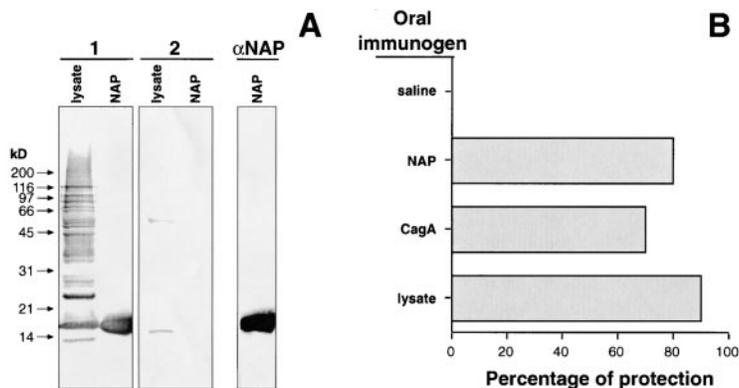
Figure 1. HP-NAP is immunogenic in humans and protective in mice. (A) The sera of an *H. pylori*–infected patient (1) and a negative control (2) were assayed by immunoblotting followed by development with enhanced chemiluminescence on samples of *H. pylori* total sonicates (left lanes) and purified recombinant HP-NAP (right lanes), subjected to SDS-PAGE, and then transferred onto nitrocellulose paper. Numbers on the left refers to the position of molecular weight markers (in kD). The last lane (αNAP) shows an immunoblot of recombinant HP-NAP stained with an anti–HP-NAP antiserum obtained from rabbits. (B) Groups of 10 CD-1 mice were immunized three times intragastrically with the indicated antigens together with the LTK63 mutant as a mucosal adjuvant, and then challenged with 10^9 CFU of *H. pylori* strain SPM326 (see Materials and Methods for details). Mice were considered protected when no colonies were counted from their stomachs.

for 5 min at 37°C; or with 5 ng/ml of TNF-α for 15 min, or IFN-γ for 30 min at 37°C. After incubation, the cells were stimulated with HP-NAP or FMLP and the production of H_2O_2 was measured.

To evaluate the effect on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), 10^6 neutrophils/ml loaded with Fura-2 were incubated for 120 min with 800 ng/ml of pertussis toxin, or for 5 min with PP1 or wortmannin at 37°C. After incubation, cells were treated with HP-NAP or FMLP and the calcium level was monitored as described above.

NADPH Oxidase Activation. The activation of NADPH oxidase was investigated by evaluating the translocation of cytosolic components p47phox, p67phox, and p40phox from the cytosol to the plasma membrane after cell stimulation, as described previously (36). In brief, neutrophils (7×10^6 cells/ml) were incubated at 37°C under stirring with or without the agonists. At the indicated time, cells were disrupted by sonication (two 15-s cycles at 200 W, 4°C). The postnuclear supernatants were loaded onto a discontinuous gradient of 1.5 ml of 15% (wt/wt) sucrose layered on 1.5 ml of 34% (wt/wt) sucrose in relaxation buffer (37, 38) and centrifuged at 150,000 *g* (45 min, 4°C). The light membranes were collected at the 15%/34% sucrose interface, washed with relaxation buffer, centrifuged at 150,000 *g* (30 min, 4°C), resuspended in 150 μl of sample buffer, and boiled for 5 min. Aliquots of samples containing the same amounts of proteins were subjected to SDS-PAGE on 12% polyacrylamide gels and incubated overnight with 1:500 diluted anti-p47phox, anti-p67phox, and anti-p40phox antibodies. All of the subsequent steps for enhanced chemiluminescence Western blotting were performed as detailed elsewhere (36). In separate samples, kept under the same conditions of incubation, the stimulation of H_2O_2 production was measured to control the effect of the agonists.

Results

HP-NAP Is an Important Antigen in the Human Immune Response. In a first set of experiments, we tested by ELISA if serum samples from individuals infected with *H. pylori* contained antibodies directed against HP-NAP, and found that 21 (60%) out of 35 subjects positive for *H. pylori* produced antibodies specific for HP-NAP. Antibodies specific for CagA, an immunodominant antigen of *H. pylori* (26, 30, 37, 38), were detected in 26 (74%) of the subjects. These data indicate that HP-NAP induces specific immune responses in humans after infection with *H. pylori*, with a

prevalence close to that of CagA. Fig. 1 A shows the result of a representative immunoblot experiment using HP-NAP$^+$ and HP-NAP$^-$ serum samples. HP-NAP is a 17-kD protein (20, 22) that runs at the gel front of low cross-linked polyacrylamide gels, which may explain why this strong anti–HP-NAP immunoreactivity has gone unnoticed in previous routine analyses of antigen specificities of the antibodies present in antisera of *H. pylori*–infected patients. A more extensive analysis of HP-NAP immunoreactivity is underway (our unpublished results); however, the present data clearly indicate that HP-NAP is an important antigen in the human immune response against *H. pylori*.

HP-NAP Induces an H. pylori Immunoprotective Response in Mice. To test whether the HP-NAP antigen plays a role in immunoprotection, groups of mice were immunized orally with HP-NAP. Saline and CagA and an *H. pylori* sonicate were used as negative and positive controls, respectively. After challenge with the *H. pylori* strain SPM326, protection was obtained in 80% of the mice immunized with recombinant HP-NAP (Fig. 1 B). The level of protection induced is comparable to that obtained with known protective antigens such as CagA and the *H. pylori* total extract. These results show that HP-NAP is a protein that confers protection in this animal model of *H. pylori* in-
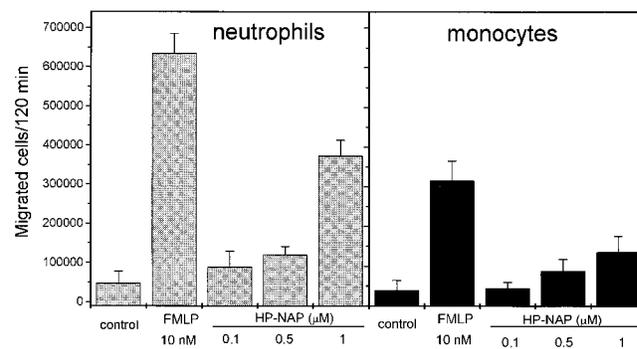

Figure 2. Dose–response stimulation of HP-NAP chemotaxis in neutrophils and monocytes. The chemotactic activity of HP-NAP on human neutrophils and monocytes is compared with that of FMLP. Chemotaxis was measured in Costar transwells as specified in the Materials and Methods. Bars represent the average of three independent experiments performed in duplicates, and the SD values are given at the top.
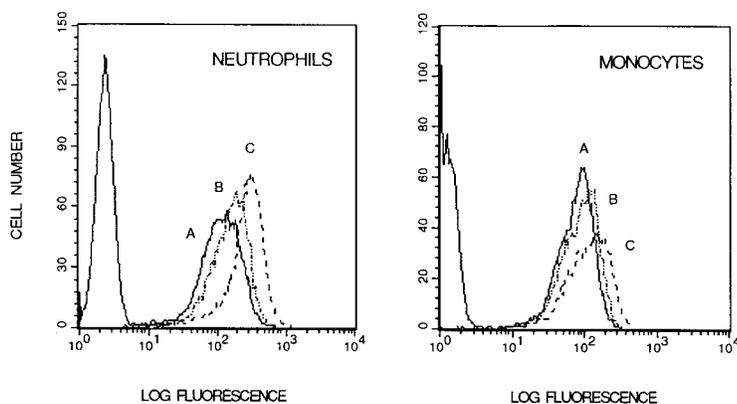
Figure 3. HP-NAP upregulates the expression of β2 integrins in neutrophils and monocytes. The HP-NAP–induced expression of the protein on the cell surface was determined by FACS® analysis with a specific mAb: (A) control cells; (B) cells treated with 0.2 mM FMLP for 30 min at 37°C; (C) cells treated with 0.5 μM HP-NAP for 30 min at 37°C.

fection, as already demonstrated with other bacterial antigens such as CagA, native and recombinant VacA, and urease (27, 39, 40).

These in vivo findings in both humans and mice, together with the notion of the existence of genes encoding for HP-NAP–like proteins in many pathogenic bacteria, prompted us to further define the biological activity of HP-NAP at the molecular and cellular levels.

HP-NAP Stimulates Chemotaxis and Integrin Expression in Monocytes and Neutrophils. The strong infiltration of neutrophils typical of *H. pylori*–associated gastritis (1–5) raises the possibility that bacterial virulence factors display chemotactic activity. Fig. 2 shows that indeed HP-NAP induces a dose-dependent stimulation of chemotactic migration of neutrophils and monocytes.

Previous reports have shown that chemoattractants such as formyl peptides, C5a, and IL-8 upregulate β2 integrin expression on polymorphonuclear leukocytes and monocytes (41–43). As this process is involved in leukocyte extravasation (41–43), the ability of HP-NAP to trigger increased expression of β2 integrin was tested with an mAb recognizing the heterodimer common β chain (CD18). This allows evaluation of LFA-1, CR3, and p150/95 expression. Fig. 3 shows that HP-NAP induces in neutrophils

and monocytes a consistent increase of β2 integrin expression, very similar to that induced by FMLP.

HP-NAP Stimulates the Production of ROI. These biological properties of HP-NAP called for a detailed analysis of their mode of activation of human leukocytes. It was previously reported that HP-NAP induces neutrophils to reduce NBT via increased production of ROI (20). Here, we have assayed quantitatively the time course of the stimulant activity of HP-NAP on both human neutrophils and monocytes (Fig. 4). The stimulation of ROI production is detectable within a few minutes after the addition of HP-NAP and remains linear for 30–40 min. HP-NAP acts in a dose-dependent manner and, at any dose, the stimulation of ROI production is slower than that induced by FMLP. However, the magnitude of effect attained at the maximal doses of the two stimulants is similar (Fig. 4). As with FMLP (44), the stimulation of ROI production by HP-NAP is potentiated about four times by cytochalasin B (5 μg/ml added 5 min before the stimulant; not shown).

HP-NAP is a dodecamer, and strongly denaturing conditions are necessary to dissociate it into monomers (25). Presumably, the neutrophil and monocyte activating form of HP-NAP is the dodecameric species, though we cannot exclude the possibility that a dissociation takes place at the
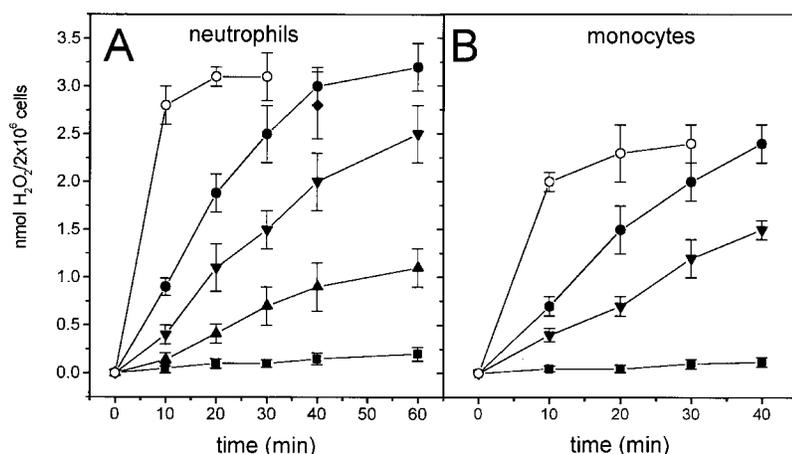

Figure 4. Stimulation of H_2O_2 production by different doses of HP-NAP in human peripheral blood neutrophils (A) and monocytes (B). H_2O_2 was measured with the homovanillic acid method (reference 34). Very similar results were obtained by assaying the reduction of cytochrome c (not shown). Points are the average of five different experiments run in triplicate, and bars represent SD values. Symbols indicate: ■, control; ◆, the data obtained with HP-NAP purified from *H. pylori* (average value of data obtained from two different preparations); all other points shown here and in the following pictures were obtained with highly purified recombinant HP-NAP expressed in *B. subtilis*: ▲, 0.6 μM HP-NAP; ▼, 1.2 μM HP-NAP; ●, 3 μM HP-NAP; ○, 1 μM FMLP.
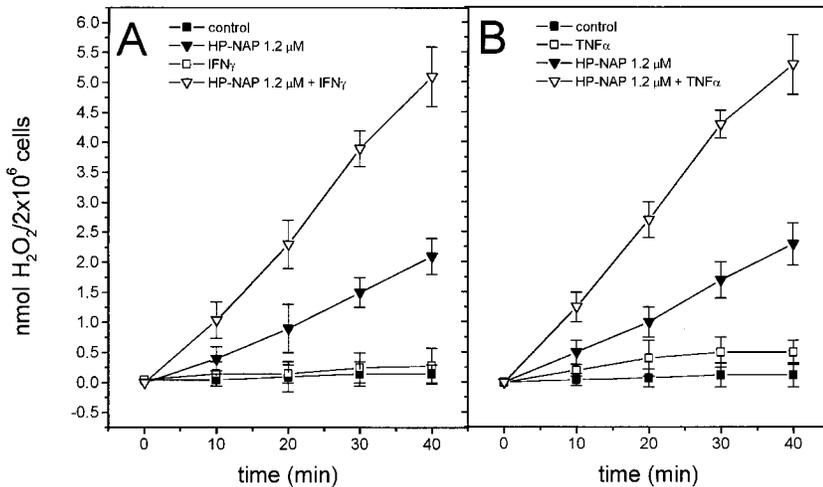
1470   *H. Pylori* Neutrophil-activating Protein Is Chemotactic and Immunogenic

Figure 5. IFN-γ (A) and TNF-α (B) potentiate the stimulation of ROI production by HP-NAP. H_2O_2 was measured with the homovanillic acid method (reference 34). Before addition of 1.2 μM HP-NAP, neutrophils were preincubated for 60 min with 5 ng/ml IFN-γ or 15 min with 5 ng/ml TNF-α at 37°C. Points are the average of three different experiments run in triplicate, and bars represent SD values.

neutrophil cell surface. Considering dodecameric HP-NAP as the active species, its effectiveness in stimulating neutrophils in molar terms is comparable to that of FMLP.

IFN-γ and TNF-α Strongly Potentiate the Effect of HP-NAP. Several mediators of the inflammatory response, including the proinflammatory cytokines TNF-α and IFN-γ, potentiate the stimulus-induced activation of the NADPH oxidase of macrophages (36, 45–48). Fig. 5 shows that TNF-α and IFN-γ effectively prime human neutrophils with respect to the production of ROI induced by HP-NAP. Similar results were obtained with monocytes (not shown). This finding is particularly relevant in the context of the chronic infection established by *H. pylori*, where a synergistic effect among HP-NAP and the cytokines may amplify the pathogenic effect of the ROI.

HP-NAP–induced ROI Production Is Mediated by NADPH Oxidase. Several cellular sources may be involved in the production of ROI stimulated by exposure of neutrophils to HP-NAP, including the mitochondrial respiratory chain, microsomal enzymes, xanthine oxidase, the arachidonic acid cascade, unidentified NADH–NADPH-dependent enzymes, and the classical plasma membrane NADPH oxidase (49, 50). The inactive form of the oxidase is present in resting phagocytes and consists of membrane-bound components (gp91phox and p22phox), the flavocytochrome b_{558}, and cytosolic components (p47phox, p67phox, p40phox, and Rac1/Rac2). The oxidase is activated by multiple signal transduction pathways, all of them causing the translocation of the cytosolic subunits to the plasma membrane, where they associate to cytochrome b_{558} and activate the univalent reduction of oxygen to superoxide anion by the cytosolic NADPH (51). To determine if the NADPH oxidase is the source of the ROI production induced by HP-NAP, we assayed for the translocation of its cytosolic components to the plasma membrane by subcellular fractionation of neutrophils and monocytes treated or not treated with HP-NAP. Fig. 6 shows that antibodies specific for p47phox, p67phox, and p40phox detect minor amounts of these proteins in the plasma membrane fraction of unstimulated neutrophils, whereas they strongly stain the same fraction

obtained from cells stimulated with HP-NAP or with the classical agonist PMA. These results imply that HP-NAP induces the translocation of the cytosolic subunits of the NADPH oxidase from the cytosol to the membrane, thereby causing its activation to produce ROI.

To further prove the central role of NADPH oxidase in the HP-NAP effect, we used neutrophils and monocytes from one CGD patient. CGD is an inherited condition where the absence of cytochrome b (X-linked form) or of p47phox or p67phox (autosomal form) results in an inactive NADPH oxidase (52). Thus, the leukocytes of CGD patients are unable to produce ROI in response to all the agonists of this enzyme. Fig. 7 shows that the peripheral blood leukocytes of a CGD patient, lacking p67phox, were unresponsive to HP-NAP in terms of ROI production.

Finally, we tested the effect of DPI on the HP-NAP stimulation of neutrophils. DPI is an inhibitor of cellular flavoproteins (53) and as such inhibits ROI produced by the NADPH oxidase. Fig. 8 shows that DPI (10 μM) strongly inhibits HP-NAP–induced ROI production, thus providing further evidence that this *H. pylori* virulence factor acts via activation of the plasma membrane NADPH oxidase complex.

The Production of ROI Induced by HP-NAP Is Inhibited by Pertussis Toxin, Wortmannin, and PP1. Activation of NADPH oxidase in phagocytes is a complex phenomenon that may or may not depend on the stimulant binding to a specific receptor. Depending on the chemical nature of the stimulus, it may implicate G proteins, second messengers,

1 2 3

p 67phox

p 47phox

p 40phox

Figure 6. Translocation of cytosolic components of NADPH oxidase on the plasma membrane in human neutrophils stimulated with HP-NAP. Lane 1, unstimulated neutrophils; lane 2, neutrophils stimulated for 15 min with 3 μM HP-NAP; lane 3, neutrophils stimulated with PMA (50 ng/ml) for 15 min. The conditions of cell stimulation, fractionation, and detection of cells components are described in Materials and Methods.
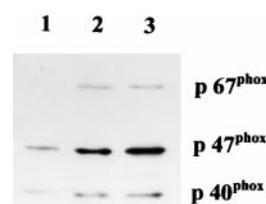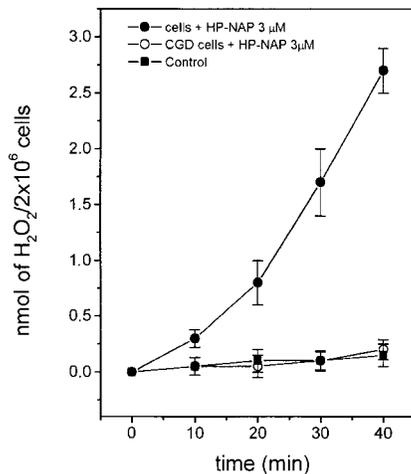
Figure 7. HP-NAP does not stimulate H_2O_2 production in CGD neutrophils defective of NADPH oxidase. Experiments were performed twice in triplicate, and H_2O_2 production was measured with the homovanillic acid method (reference 34). Bars represent the SD value.

activation of protein kinases, and phospholipid turnover (49, 51). Fig. 8 shows that pertussis toxin and wortmannin are powerful inhibitors of the HP-NAP–induced ROI production by neutrophils. Pertussis toxin ADP-ribosylates the Gα subunits of heterotrimeric G proteins of the Gi and Go subtypes, which are coupled to heptahelical receptors and activate phosphatidylinositol-specific phospholipase C (PtdIns-PLC) (54–56). The present finding is a clear demonstration that one such regulatory GTP-binding protein mediates the transmission of the HP-NAP signal from the cell surface to the cell interior. At the same time, it implicates both a heptahelical HP-NAP–specific receptor and a PtdIns-PLC, which accounts for the generation of the 1,4,5-trisphosphate agonist of the calcium channel present on the limiting membrane of intracellular calcium stores.

The inhibitory effect of wortmannin is exerted within a nanomolar range of concentrations at which this drug is specific for PI3-K and has no toxic effects on human neutrophils (57, 58). This is indirect but compelling evidence that the HP-NAP activation of neutrophils, mediated by a heterotrimeric G protein, causes a downstream activation of a PI3-K.

PP1 is a specific inhibitor of a family of Src-selective tyrosine kinases (59) that is implicated in NADPH oxidase activation exerted by some stimulants. Fig. 8 shows that this is also the case for HP-NAP, indicating that receptor binding of this stimulant activates a pathway implicating phosphorylation of tyrosine residues.

HP-NAP Induces an Increase in Cytosolic [Ca^{2+}]. The activation of NADPH oxidase by receptor-mediated agonists such as C5a, platelet activating factor (PAF), and FMLP is associated with an increase in cytosolic [Ca^{2+}] released from intracellular stores and influxed from the extracellular medium (60). Fig. 9 shows that HP-NAP induces an increase of cytosolic calcium concentration in a dose-dependent manner. When the stimulation was performed
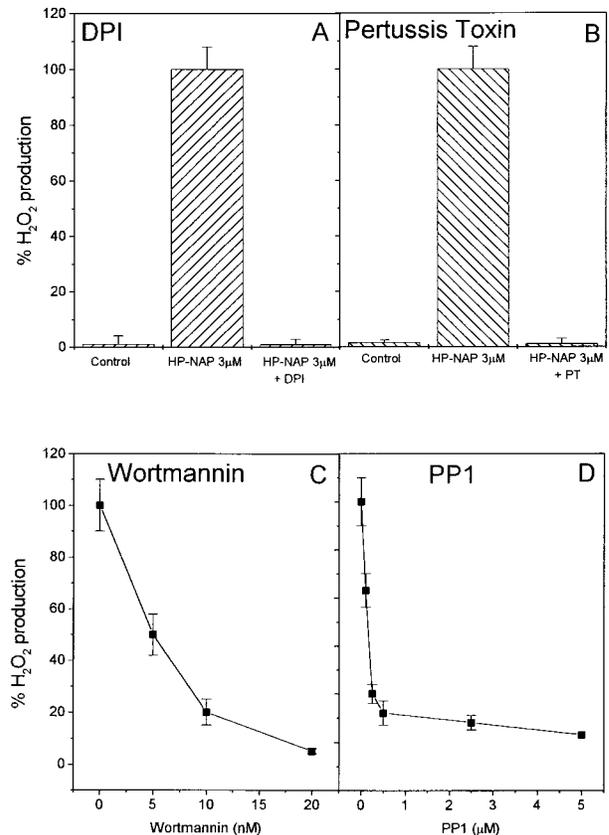

Figure 8. Effect of DPI (A), pertussis toxin (B), wortmannin (C), and PP1 (D) on the stimulation of H_2O_2 production by HP-NAP. Human neutrophils were preincubated with the indicated inhibitor at the concentrations reported in the figure for 5 min at 37°C (2 h for pertussis toxin), before addition of 3 μM HP-NAP, and then assayed as in the legend to Fig. 5. Data are the average of the results of two different experiments run in triplicate, and bars represent SD values.

in the absence of external calcium, the [Ca^{2+}]$_i$ was halved, indicating an approximately similar contribution of extracellular and intracellular Ca^{2+}. The increase in cytosolic [Ca^{2+}] caused by HP-NAP was completely prevented by pretreatment with pertussis toxin (Fig. 9 C). This result implies that the G protein activated by HP-NAP stimulates a PLC, which releases 1,4,5-trisphosphate, which in turn opens intracellular calcium channels, thus leading to the increased cytosolic calcium level documented here. On the contrary, the effect of HP-NAP on cytosolic [Ca^{2+}] was unaffected by inhibitors of Src tyrosine kinase (PP1) and phosphatidylinositol 3,4,5-trisphosphate kinase (wortmannin) (Fig. 9 C).

In the presence of the intracellular Ca^{2+} chelator BAPTA (60 μM), HP-NAP was not able to stimulate the production of ROI (not shown). The effect of BAPTA was not due to a toxic activity, as cells loaded with this chelator maintained their responsiveness to PMA (data not shown). These results indicate that the increase in [Ca^{2+}]$_i$ plays a functional role in the sequence of events causing the stimulation of NADPH oxidase by HP-NAP, as it occurs with chemotactic peptides (60).
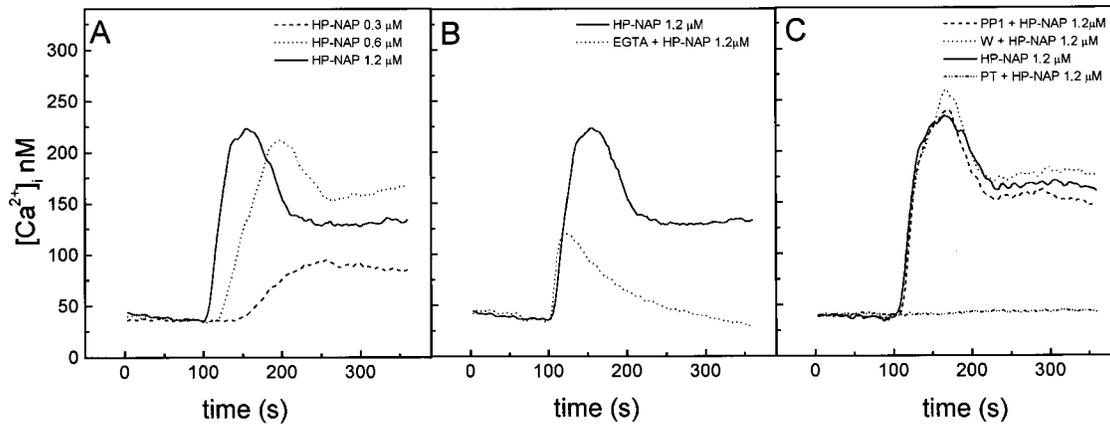
1472   *H. Pylori* Neutrophil-activating Protein Is Chemotactic and Immunogenic

Figure 9. Effect of HP-NAP on $[Ca^{2+}]_i$ in neutrophils in the absence or presence of pertussis toxin, wortmannin, PP1, and EGTA. (A) Human neutrophils loaded with Fura-2 for 25 min were washed and then exposed to the given concentrations of HP-NAP in a stirred fluorimeter cuvette. Fluorescence outputs were converted to $[Ca^{2+}]$ after calibration. (B) Only part of the increase in cytosolic $[Ca^{2+}]$ triggered by HP-NAP (solid line) is due to release from intracellular stores (broken line). (C) Pertussis toxin completely prevents the HP-NAP–induced rise in cytosolic $[Ca^{2+}]$, whereas other inhibitors are ineffective.

Discussion

The main findings of this work are that HP-NAP produced by *H. pylori*: (a) is a major antigen in the human immune response to *H. pylori*; (b) is capable, after oral immunization, of conferring protection on mice against a subsequent challenge with *H. pylori*; (c) is a chemoattractant for neutrophils and monocytes; and (d) activates the NADPH oxidase of human leukocytes via a set of signaling events that are defined here. Moreover, here we provide quantitative assays of HP-NAP that are essential for evaluating the biological and pathological activity of HP-NAP derivatives and mutants as well as of HP-NAP–like molecules produced by other bacteria.

Many studies have been devoted to the definition of the immunogenic properties of *H. pylori* molecules (16, 26, 27, 38–40). CagA has been considered as an immunodominant antigen, since anti-CagA antibodies are frequently detected in sera from individuals infected with CagA$^+$ *H. pylori* strains (30, 61, 62), and CD4$^+$ T cell clones isolated from the gastric mucosa of *H. pylori*–infected subjects are frequently specific for CagA (63). Antibodies specific for the *H. pylori* urease, heat shock proteins, VacA, and other antigens are also found in sera from *H. pylori*–infected patients (38–40, 60, 64). Here, we have shown that *H. pylori*–infected patients produce antibodies specific for HP-NAP. It is not yet known what role these antibodies have during the course of *H. pylori* infection. However, it is particularly interesting to note that oral administration of HP-NAP in association with a strong and nontoxic mucosal adjuvant is capable of protecting mice against a challenge with *H. pylori*. Even though the mechanisms of the inflammatory and immune response to *H. pylori* infection are not yet fully understood, we believe that these are major findings with respect to the definition of the virulence factors involved in the pathogenesis of *H. pylori*–associated diseases and to the formulation of an anti–*H. pylori* vaccine.

It was previously shown that HP-NAP promotes neutrophil adhesion to endothelial cells and oxygen radical–mediated reduction of NBT (19, 20). Here we have shown that HP-NAP is chemotactic for both monocytes and neutrophils and we have identified the signaling events involved in their activation. The conclusion that ROI production is due to the activation of the classical $O_2 \cdot^-$ forming NADPH oxidase is supported by three lines of evidence. HP-NAP stimulation of ROI production is associated with the translocation of the NADPH oxidase cytosolic components p47^{phox}, p67^{phox}, and p40^{phox} to the plasma membrane of neutrophils, a process that corresponds to the activation of the oxidase (65). Second, leukocytes of CGD patients, genetically defective in NADPH oxidase, do not respond to HP-NAP. Third, DPI, an inhibitor of NADPH oxidase, prevents the stimulus provided by HP-NAP. After establishing this point, we attempted to define the intracellular signaling pathways involved in such activation.

It is known that various reactions participate in NADPH oxidase activation; some of them are always activated regardless of the nature of the stimulant (49–51). Here, we have shown that the HP-NAP activation of neutrophil ROI production is consistent with a signaling pathway involving a pertussis toxin–sensitive G protein, an elevation of cytosolic $[Ca^{2+}]$, a Src family of tyrosine kinases sensitive to PP1, and a wortmannin-sensitive PI3-K. These intracellular signaling events result in an HP-NAP–induced chemotactic response with increased expression of β2 integrin in both neutrophils and monocytes.

The pattern of events triggered by HP-NAP is closely similar to that triggered by heptahelical receptors specific for the chemotactic agonist FMLP, C5a, platelet activating factor (PAF), and IL-8 (49, 51). Such similarity also strongly suggests that the HP-NAP receptor is a serpentine type of cell surface transmembrane protein, but this receptor remains to be identified.

The fact that HP-NAP is a chemotactic agonist and an activator of the oxidative metabolism of the inflammatory cells is relevant for the pathogenic effect of *H. pylori* at the sites of infection. In fact, HP-NAP can be involved in the extravasation of leukocytes (gastritis), and ROI, apart from being proinflammatory mediators (66, 67), could play a role in the damage of the stomach mucosa during chronic *H. pylori* infection. ROI may also be involved in the generation of mutagenic chemical species that can contribute to the pathogenesis of *H. pylori*–associated stomach cancers (68, 69). HP-NAP has to be considered an important novel virulence factor of *H. pylori*–associated disease whose role in bacterial physiology remains to be established. Regarding its role in pathogenesis, it is noteworthy that there is a correlation between severity of disease and capability of activating neutrophils of different *H. pylori* isolates (19, 20). We also report here that TNF and IFN potentiate the HP-NAP–induced activation of the NADPH oxidase. Such priming effects are expected to be relevant to the in vivo situation, where these proinflammatory cytokines are likely to potentiate the stimulation of ROI production by HP-NAP and consequently their pathogenic effect.

The findings presented here make HP-NAP a strong candidate for a multicomponent *H. pylori* vaccine. However, on the basis of the results obtained with human leukocytes, it should be clarified whether HP-NAP needs to be modified in such a way as to lose its phagocyte-stimulating activity while preserving its immunogenicity, or whether the stimulatory properties are prerequisites for optimal stimulation of the immune response. The region(s) of HP-NAP involved in the interaction with cell surface receptor(s) remain(s) to be identified; such information may be acquired via generation of site-directed mutants, designed from recent structural data (25), which are underway. The present definition of quantitative assays of its biological activities and the information on the cellular signaling pathways activated by HP-NAP are prerequisites for the careful evaluation of the properties of such HP-NAP mutants.

Note added in proof. Kimmel et al. reported that six out of six gastritis patients had anti–HP-NAP antibodies (Kimmel, B., A. Bosserhoff, R. Frank, R. Gross, W. Goebel, and D. Beier. 2000. *Infect. Immun.* 68:915–920).

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