Infiltration of neutrophils and monocytes into the gastric mucosa is a hallmark of chronic gastritis caused by *Helicobacter pylori*. Certain *H. pylori* strains nonopsonized stimulate neutrophils to production of reactive oxygen species causing oxidative damage of the gastric epithelium. Here, the contribution of some *H. pylori* virulence factors, the blood group antigen-binding adhesin BabA, the sialic acid-binding adhesin SabA, the neutrophil-activating protein HP-NAP, and the vacuolating cytotoxin VacA, to the activation of human neutrophils in terms of adherence, phagocytosis, and oxidative burst was investigated. Neutrophils were challenged with wild type bacteria and isogenic mutants lacking BabA, SabA, HP-NAP, or VacA. Mutant and wild type strains lacking SabA had no neutrophil-activating capacity, demonstrating that binding of *H. pylori* to sialylated neutrophil receptors plays a pivotal initial role in the adherence and phagocytosis of the bacteria and the induction of the oxidative burst. The link between receptor binding and oxidative burst involves a G-protein-linked signaling pathway and downstream activation of phoshatidylinositol 3-kinase as shown by experiments using signal transduction inhibitors. Collectively our data suggest that the sialic acid-binding SabA adhesin is a prerequisite for the nonopsonic activation of human neutrophils and, thus, is a virulence factor important for the pathogenesis of *H. pylori* infection.

Colonization of the human stomach with *Helicobacter pylori* is accompanied by chronic active gastritis, which may lead to peptic ulcer disease, atrophic gastritis, and gastric adenocarcinoma (1). To date a number of *H. pylori* virulence factors have been identified. Among these are the urease, the blood group antigen-binding adhesin (BabA), the cag pathogenicity island, the vacuolating cytotoxin (VacA), and the *H. pylori* neutrophil-activating protein (HP-NAP). Binding of the bacterium to fucosylated host cell receptors is mediated by the BabA adhesin, an outer membrane protein of *H. pylori* (2). The cag pathogenicity island encodes a type IV secretion system that enables translocation of the CagA protein into host cells, where the protein becomes tyrosine-phosphorylated and subsequently activates a eukaryotic phosphatase leading to dephosphorylation of host cell proteins and morphological changes (3). The VacA toxin induces formation of large cytoplasmic vacuoles in eukaryotic cells and causes alterations of tight junctions (4). VacA also forms anion-selective channels, which may be blocked by chloride channel inhibitors (5, 6). HP-NAP promotes the adhesion of neutrophils to endothelial cells and the production of reactive oxygen radicals (4, 7).

A prominent feature of the *H. pylori*-induced gastritis is an infiltration of neutrophils into the gastric epithelium (8). Neutrophils play a major role in epithelial injury, because these cells have direct toxic effects on the epithelial cells by releasing reactive oxygen and nitrogen species and proteases (9, 10). An additional virulence factor of *H. pylori* bacterial cells is thus the neutrophil-activating capacity, i.e. the ability of certain *H. pylori* strains to activate human neutrophils in the absence of opsonins (8). Strains with neutrophil-activating capacity are significantly more often isolated from patients with peptic ulcer disease (8, 11, 12). The factor(s) of *H. pylori* responsible for the activation of neutrophils are heat-labile and dependent on whole nondisintegrated organisms (8). Recently, preincubation with sialylated oligosaccharides demonstrated that the nonopsonic *H. pylori*-induced activation of human neutrophils occurs by lectinophagocytosis, i.e. recognition of sialylated glycoconjugates on the neutrophil cell surface by a bacterial adhesin leads to the phagocytosis and the oxidative burst reactions (13).

To date, two sialic acid-binding proteins of *H. pylori* have been characterized: the sialic acid-binding adhesin SabA and the NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAc-binding neutrophil-activating protein HP-NAP (14, 15). SabA is the sole factor responsible for binding of *H. pylori* bacterial cells to gangliosides, as recently demonstrated by using knock-out mutant strains devoid of the SabA adhesin or HP-NAP (16). A number of sialylated *H. pylori*-binding glycopshingolipids have been identified: sialyl-α3-neoctotetraosylceramide, sialyl-α3-neoctohexaosylceramide, sialyl-α3-neoctoctaoxsylceramide, the VIM-2 ganglioside and sialyl-dimeric-Leα glycosphingolipid (14, 16–18). Several of these *H. pylori*-binding gangliosides are neutrophil-activating protein of *H. pylori*; BabA, sialic acid-binding adhesin; VacA, vacuolating cytotoxin of *H. pylori*; wt, wild type; PBS, phosphate-buffered saline; GM1, Galβ3GalNAcβ3( NeuAcα3Galβ4Glcβ1Cer. **
also present in human neutrophils (19, 20). In addition, some *H. pylori* strains bind to polyglycosylceramides and glycoproteins of human neutrophils in a sialic acid-dependent binding manner (21).

In the present study the role of some *H. pylori* virulence factors in the nonsonic *H. pylori*-induced activation of human neutrophils was investigated. Human neutrophils were challenged with wild type *H. pylori* strains and isogenic deletion mutant strains lacking HP-NAP, BabA, Saba, VacA, or the 37-kDa fragment of VacA, followed by chemiluminescence measurement of the superoxide anions produced by the neutrophils. The nonsonic adherence to and phagocytosis by neutrophils of wild type and mutant bacterial cells were examined at various time intervals as the appearance of viable macroscopic aggregation/agglutination of neutrophils and by microscopy of acridine orange stained smears. In addition, the effects of signal transduction inhibitors on *H. pylori*-induced neutrophil activation were studied, to identify intracellular signaling pathways required for *H. pylori*-induced neutrophil oxidative burst.

**MATERIALS AND METHODS**

*H. pylori* Strains, Culture Conditions, and Labeling—Characteristics of the *H. pylori* strains are presented in Table I. Strain NCTC 11637 was obtained from the National Collection of Type Cultures, London, UK, strain C-7050 from Professor T. Kosunen, Helsinki, Finland, and strain CCUG 17874 from the Culture Collection University of Göteborg, Sweden. Strain J99 and the construction of the J99/Saba mutant saba::JHFP662:cam were described by Mahdavi et al. (14). The J99/BabA mutant (baba::cam) and the J99/BabA::Saba mutant (baba::cam saba::kan) were constructed as previously described (2, 14).

For construction of the HP-NAP knock-out mutant, designated J99/ NapA3F and NapA4R which verified that the KanR cassette was inserted in the napA gene was amplified by PCR using the napA1F (forward) and napA1R (reverse) primers. The PCR fragment was cloned into the pBluescriptSK+ vector (Stratagene, La Jolla, CA). The resulting plasmid was linearized with primers napA2F (forward) and napA1R (reverse) primers. The PCR fragment was cloned into the pBlueScriptSK+ EcoRV site (Stratagene, La Jolla, CA). The resulting plasmid was linearized with primers napA3F and napA4R which verified that the KanR cassette was inserted into napA. Western blot analysis of napA mutants using anti-HP-NAP antibodies showed that the mutant strain was devoid of HP-NAP expression.

The oligonucleotides used for PCR were: napA1F, 5'-TCAAGCCCAT-AGCGGATAAGCT-3'; napA1R, 5'-TGGTGTAGGATAGCGAT-3'; napA2F, 5'-CACGATCGCATCCGCTTGCA-3'; napA2R, 5'-TTACCG-TGAATCTTGGCAGTTAG-3'; napA3F, 5'-TGTTGGTAGACGCGATGCAG-3'; and napA4R, 5'-TAAATGCTACCTCAGTCTAGAAGC-3'. TheVacA knock-out mutant (17874/VacA--) and the mutant with deletion of the 37 kDa fragment of VacA (17874/p37--) were constructed as previously described (23).

For chromatogram binding experiments the bacteria were grown in a microaerophilic atmosphere at 37 °C for 48 h on Brucella medium. The bacteria were grown on agar plates before transfer to liquid culture. The agar plates were supplemented with 1% bovine serum (BBL), 10% horse serum, and 1% IsoVitaleX enrichment (BBL), without antibiotics for the wild type strains, and with antibiotics as above for the mutant strains J99/Saba-- J99/BabA--, J99/BabA--Saba-- 17874/ VacA-- and 17874/p37--. The *H. pylori* organisms were collected in PBS and used in chemiluminescence and phagocytosis experiments as described below.

**Chromatogram Binding Assay—**Glycosphingolipids were isolated and characterized by mass spectrometry, 1H NMR, and degradation studies, as described (24). De-sialylation was done by incubating the glycosphingolipids in 1% acetic acid (by volume) at 100 °C for 1 h.

Thin-layer chromatography was performed on glass- or aluminum-backed silica gel 60 HPTLC plates (Merck, Darmstadt, Germany). Mixtures of glycosphingolipids (40 μg) or pure compounds (1-4 μg) were dissolved using chloroform/methanol/water (60:35:8, by volume) as solvent system. Chemical detection was accomplished with anisaldehyde (25).

Binding of 35S-labeled *H. pylori* to glycosphingolipids on thin-layer chromatograms was done as previously reported (26). Dried chromatograms were dipped for 1 min in diethylether/hexane (1:5, by volume) containing 0.5% (w/v) polysorbuthemacylate (Aldrich). After drying, the chromatograms were soaked in PBS containing 2% bovine serum albumin (w/v), 0.1% NaN3 (w/v), and 0.1% Tween 20 (by volume) for 2 h at room temperature. The chromatograms were subsequently covered with radiolabeled bacteria diluted in PBS (2-5 × 106 cpm/ml). Incubation was done for 2 h at room temperature, followed by repeated washings with PBS. The chromatograms were thereafter exposed to XAR-5 x-ray films (Eastman Kodak, Rochester, NY) for 12 h.

**Extraction of Membrane Proteins from Human Neutrophil Granulocytes—**Membranes from fresh neutrophils were isolated as described previously (27). The outer membrane fragment fraction was dissolved in 25 mM Tris-HCl containing 2.5% SDS and 1 mM EDTA, pH 8.0, heated to 95 °C for 10 min and centrifuged at 10,000 × g for 10 min. 

**Electrophoresis and Binding of *H. pylori*—**SDS-PAGE and staining were carried out with NuPAGE3® gels (Novex, San Diego, CA). Briefly, the membrane protein samples in St Gene buffer with 50 mM dithiothreitol were added, heated to 95 °C for 5 min, and applied on a homogeneous 10% polyacrylamide gel. After electrophoresis, the gels were either stained with Coomassie Blue or electroblotted to polyvinylidene difluoride (0.2-μm) membranes.

The polyvinylidene difluoride membrane was incubated in blocking solution, 3% bovine serum albumin, 50 mM Tris-HCl, 200 mM NaCl, 0.1% SDS, pH 8.0, for 1 h at room temperature. The membrane was then incubated with 35S-labeled *H. pylori* strain CCUG 17874 diluted in PBS for 2 h at room temperature and thereafter washed in a solution of 50 mM Tris-HCl, 200 mM NaCl, and 0.05% Tween 20, pH 8.0. After drying at room temperature, the membrane was exposed to XAR-5 x-ray films overnight. Reference bovine fetuin and bovine asialofetuin were purchased from Sigma.

**Human Neutrophil Granulocytes—**Heparinized blood from healthy blood donors was used to prepare neutrophils by Ficoll-Paque (Amer sham Biosciences) centrifugation in accordance with the method of Boyum (28), slightly modified as described (8). For each series of experiments on a particular day neutrophils were prepared and pooled from three blood donors of the same blood group (A Rh+ or O Rh+). Neutrophils were thus obtained from different blood donors at each experiment. They were suspended in PBS supplemented with MgCl2, CaCl2, glucose, and gelatin as previously described (8). The purity and viability of the neutrophils exceeded 95%.

**Chemiluminescence Experiments—**The oxidative burst of neutrophils challenged with nonsonopsonized whole and live *H. pylori* organisms was measured with luminol enhanced chemiluminescence (CL) as previously described (8). To each test tube (LKB, Bromma, Sweden) were added 300 μl of PBS supplemented with MgCl2 and CaCl2, 100 μl of neutrophils (5 × 10^6/ml), 50 μl of 10-5 M luminol (Sigma), and finally 50 μl of nonsonopsonized *H. pylori* (5 × 10^6/ml).

For the signal transduction inhibition experiments neutrophils (5 × 10^6/ml) were treated with 800 ng of pertussis toxin for 60 or 120 min at 37 °C in serum-free medium (5, 10, or 20 μg) for 5 min at 37 °C, or diphenylhydantoin chloride (DPI, 10 μM) for 5 min at 37 °C, centrifuged, and resuspended in PBS supplemented with MgCl2 and CaCl2 before they were challenged with *H. pylori* cells of strain NCTC 11637 as described above. Pertussis toxin, wortmannin, and DPI were purchased from Calbiochem. For oligosaccharide inhibition experiments 50 μl of *H. pylori* (5 × 10^6/ml) were mixed with 50 μl of 3'-sialyllactose (IsoSep, Tullinge, Sweden) to receive final concentrations of 0.1-1.0 μM in the
CL, for 15 min at 37 °C, and 100 µl of the mixture was thereafter transferred to the test tube for CL measuring.

The oxidative bursts of the neutrophils were measured as luminol-enhanced chemiluminescence with a luminometer (LKB Wallac 1251, Turku, Finland), and the measurements were always started within 1 min after the bacterial suspension had been added. The assays were performed at 37 °C, and CL from each sample was measured at 60- to 90-s intervals during a period of 30–60 min, and data were stored in a computer for computerized calculations. This technique thus measures both the external and internal oxidative bursts of the nonsonicophilic phagocytosis by neutrophils, which was previously checked by quenching the external burst in the presence of catalase (2000 units/ml), and the internal one in the presence of azide (1 mm) and horseradish peroxidase (4 units/ml) as described by Lock and Dahlgren (29). The H. pylori strains NCTC 11637 (giving a strong and rapid CL response) and C-7050 (inducing no CL response) (8) were included in each series of experiments as positive and negative controls, respectively.

Adherence, Phagocytosis, and Neutrophil Agglutination Assays—To each test tube were added 350 µl of PBS supplemented with MgCl₂ and CaCl₂, 100 µl of neutrophils (5 × 10⁵/ml), and 50 µl of nonopsonized H. pylori organisms (5 × 10⁶/ml). Adherence to and phagocytosis by neutrophils of wild type and mutant H. pylori strains were examined by microscopy (see below) at the various time intervals of <2–5, 20–30, and 60–90 min, and for the appearance of visible, i.e., macroscopic agglutination (aggregation) of neutrophils by ocular inspection of the tubes at the same time intervals. For microscopic examination of adherence/phagocytosis assays, and the formation of neutrophil agglutinates/aggregates by H. pylori cells, 10 µl of the H. pylori/neutrophil mixture was smeared on a glass slide within an area of ∼2.5–3 mm², air-dried, fixed in cold methanol for 5 min, washed in distilled water, and then stained with acridine orange as described by the manufacturer. The slides were inspected with a Zeiss fluorescence microscope for light spot with appropriate filter combinations at a magnification of 400× to look for bacteria that had adhered to neutrophils and/or were phagocytosed. The results obtained by this technique corresponded well to previously reported findings by electron microscopy (30). However, even though adherence and phagocytosis by the same orange-stained bacteria of H. pylori to the neutrophil cell membrane looks different from those that are obviously inside the cell, phagocytosis of an individual bacterial cell cannot be definitely separated from adherence with this technique. Adherence/phagocytosis have therefore been taken together and were graded as negative (−) when neutrophils were evenly dispersed with only occasionally adhered H. pylori cells; minor (+) or moderate (++) adherence/phagocytosis with 5 < 10 or 10 < 20 bacterial cells, respectively, per neutrophil in representative fields of view; and heavy (+++) adherence/phagocytosis with >20 bacterial cells per neutrophil.

In the signal transmission inhibition experiments, neutrophils were treated with pertussis toxin,wortmannin, and DPI in concentrations given above, and adherence to and phagocytosis by neutrophils of strain NCTC 11637 as visible macroscopic agglutination were examined at the time intervals <2–5, 20–30, and 60–90 min, and compared with untreated neutrophils challenged with the same strain. Adherence, phagocytosis, and neutrophil agglutination were graded as described above.

RESULTS

Binding of H. pylori to Human Neutrophil Gangliosides Is Lost after Deletion of the SabA Adhesin—The sialic acid binding capacities of the wild type and deletion mutant H. pylori strains utilized in this study were evaluated by binding of the bacteria to glycosphingolipids separated on thin-layer chromatograms. The results are exemplified in Fig. 1 and summarized in Table I. The criterion used for sialic acid recognition was binding to the acid glycosphingolipid fraction of human neutrophils (Fig. 1, lane 2) with no binding after de-sialylation (Fig. 1, lane 3). Thus, while both the parent strains and their mutants bound to the nonacid reference glycosphingolipid gangliotetraosylceramide (Fig. 1, lane 1), binding to human neutrophil gangliosides was observed for all strains except the J99/SabA− mutant (Fig. 1C), the J99/BabA−SabA− mutant (not shown), and the C-7050 strain (not shown).

H. pylori Also Bind to Human Neutrophil Membrane Proteins—Binding of Saba-expressing H. pylori strain CCUG 17874 to human neutrophil membrane proteins is shown in
The reduction of the CL responses obtained after incubation of the wild type *H. pylori* strains with 3\(^{-}/\)H11032-sialyllactose also showed some strain to strain variation. As an example, for strain NCTC 11637, which exhibited a very strong CL response, 0.1 mM 3\(^{-}/\)H11032-sialyllactose gave a 62% reduction of the peak value and 1 mM gave a 72% reduction (Fig. 3), and for strain J99, with a weaker response, 1 mM 3\(^{-}/\)H11032-sialyllactose totally abolished the peak (data not shown).

As shown in Fig. 4, the absence of the neutrophil-activating protein HP-NAP (J99/NAP−; Fig. 4A), or the Le\(^b\)-binding BabA adhesin (J99/BabA−; Fig. 4B), had no effect on the neutrophil activation, i.e. the CL response of the mutant strains were very similar to the response induced by the J99 parent strain. However, after deletion of the gene coding for the sialic acid-binding SabA adhesin (J99/SabA− and J99/BabA−SabA−; Fig. 4B), and when using the SabA negative C-7050 wt strain (Fig. 4A), no CL responses were obtained, demonstrating that binding of *H. pylori* to sialylated neutrophil receptors plays a pivotal initial role in the induction of the oxidative burst.

Absence of the VacA Cytotoxin Abrogates the Extracellular \(H_2O_2\) Production—Deletion of the whole *vacA* in the strain CCUG 17874 resulted in a delayed and reduced CL response (Fig. 5). The same effect was observed when the 37-kDa fragment of VacA was deleted. In both cases the initial external burst reaction was lacking, and thus no activation of the plasma membrane pool of NADPH oxidase occurred.

Adherence, Phagocytosis, and Neutrophil Agglutination Are Impaired upon Deletion of *S. mutans*—When neutrophils are challenged with nonopsonized wild type *H. pylori* strain C-7050 (C-7050) and strain J99 (J99 wt) and its isogenic mutants with deletions of HPNAP (J99/NAP−), the BabA adhesin (J99/BabA−), the SabA adhesin (J99/SabA−), and both the BabA adhesin and the SabA adhesin (J99/BabA−SabA−). The luminol enhanced chemiluminescence assay was done as described under “Materials and Methods.”

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are obvious (8, 30). This is also typical for other type I H. pylori cells challenging neutrophils. In contrast bacterial cells of the control strain C-7050, a type 2 variant (VacA\(^{-}\)/H11002/cagA\(^{-}\)/H11001) that is devoid of SabA, remain evenly dispersed among the neutrophils for more than 120 min (8). All these events seen by microscopy correspond well to the oxidative burst responses detected by luminol enhanced CL.

The results from microscopy of neutrophils challenged with wild type and mutant H. pylori strains are summarized in Table II. Thus, the mutant strains with deletions of the BabA (J99/BabA\(^{-}\)/H11002) or HP-NAP (J99/NAP\(^{-}\)/H11002) adhered to neutrophils, agglutinated them, and were phagocytosed in a manner indistinguishable from the parental wild type strains J99, NCTC 11637, and CCUG 17874. In contrast, the sialic acid binding-defective mutants J99/SabA\(^{-}\)/H11002 and J99/BabA/SabA\(^{-}\)/H11002 caused, like C-7050, no neutrophil agglutination, and only occasional bacterial cells adhered to or were phagocytosed by the neutrophils, the majority of which were evenly distributed.

The bacterial cells of the 17874/VacA\(^{-}\)/H11002 mutant, with deletion of the entire vacA gene, adhered to neutrophils and were phagocytosed. However, these events were retarded as compared with its parent strain, and no macroscopic agglutination was visible even though relatively large aggregates of adhered neutrophils were seen by microscopy. The picture was very similar for the 17874/p37\(^{-}\) mutant.

Effects of Signal Transduction Inhibitors on H. pylori-induced Neutrophil Activation—To investigate the intracellular signaling mechanisms activated by binding of H. pylori to sialylated neutrophil cell surface glycoconjugates and involved in NADPH oxidase activation, the effects of the signal transduction inhibitors DPI, pertussis toxin, and wortmannin on the chemiluminescence responses were tested. Strain NCTC 11637 was selected for the challenge of neutrophils pretreated with the signal transduction inhibitors as described under “Materials and Methods.”

The expected H. pylori responses, both the initial phase and the late phase, were decreased severalfold by treatment of the neutrophils with DPI (Fig. 6A), an inhibitor of cellular flavoproteins (32), demonstrating that the induction of the neutrophil respiratory burst by H. pylori bacterial cells occurs through assembly of both plasma membrane and the intracellular NADPH oxidases.

Next the effect of pertussis toxin, a potent inhibitor of heterotrimeric G-proteins, was evaluated. When the neutrophils were pretreated with pertussis toxin, a complete abrogation of the H. pylori-induced oxidative burst was obtained (Fig. 6B), demonstrating that the neutrophil activation induced by bind-
Table II
Adherence/phagocytosis by neutrophils of wild type and mutant H. pylori strains

| H. pylori strain | Adherence/phagocytosis as observed by microscopy | Visible macroscopic neutrophil agglutination |
|------------------|--------------------------------------------------|-----------------------------------------------|
|                  | 20–30 min                                      | 60–90 min                                    | 20–30 min                                      | 60–90 min                                      |
| NCTC 11637       | + + +                                           |                                              | Yes                                           | Yes                                            |
| C-7050           | –                                               |                                              | No                                            | No                                             |
| J99 wt           | + + +                                           |                                              | Yes                                           | Yes                                            |
| J99/NAP−         | + +                                              |                                              | Yes                                           | Yes                                            |
| J99/BabA−        | + + +                                           |                                              | No                                            | No                                             |
| J99/SabA−        | (+)                                             |                                              | No                                            | No                                             |
| J99/BabA−/SabA−  | –                                               |                                              | Yes                                           | Yes                                            |
| CCUG 17874 wt    | + +                                              |                                              | No                                            | No                                             |
| 17874/VacA−      | +                                               |                                              | No                                            | No                                             |
| 17874/p37        | +                                               |                                              | No                                            | No                                             |

*a Grading: +++, heavy adherence/phagocytosis (>20 bacterial cells/neutrophil); +: moderate adherence/phagocytosis (10 ≤ 20 bacterial cells/neutrophil); +: minor adherence/phagocytosis (5 < 10 bacterial cells/neutrophil); −: no or occasional adherent bacteria (<5 bacterial cells/neutrophil).

The neutrophils and bacteria were examined by fluorescence microscopy of acridine orange-stained smears after 20–30 and 60–90 min, respectively, as described under "Materials and Methods." The results represent finding in at least 5 of 15 representative fields of view. The occurrence in test tubes of visible macroscopic neutrophil agglutination within the same time frames is indicated by “Yes” or “No.”

The role of phosphatidylinositol 3-kinase was investigated using wortmannin, an inhibitor of this kinase. As shown in Fig. 6C, the H. pylori-induced activation of neutrophils was inhibited by wortmannin in a dose-dependent manner.

Pre-treatment of neutrophils with DPI, pertussis toxin, or wortmannin did not prevent adherence of H. pylori, which, however, was delayed and retarded when compared with untreated neutrophils (Table III). There was no obvious phagocytosis, and those H. pylori cells that adhered to the neutrophil cell membrane seemed to contribute to the occurrence of minor neutrophil aggregates.

DISCUSSION

The human pathogens H. pylori and Neisseria gonorrhoeae are the two most studied of the relatively few bacterial species that nonopsonized activate neutrophils to an oxidative burst with the production of potentially reactive oxygen species (8, 11, 33). For gonococci proteins belonging to a family of heat-modifiable outer membrane proteins termed opacity-associated proteins are responsible for the activation (34). For H. pylori several soluble factors involved in the nonopsonic neutrophil activation have been described, including HP-NAP (7), the urease (35), a low molecular weight factor in water extracts from the bacteria (36), and the cecropin-like bactericidal peptide Hp (2–20) (37).

In this study we show that the sialic acid-binding SabA adhesin of particular H. pylori strains has a pivotal role in the nonopsonic activation of human neutrophils. This is further supported by the fact that treatment of neutrophils with sialidase abrogates the activation induced by H. pylori (data not reproduced). Thus, the nonopsonic neutrophil oxidative burst induced by H. pylori bacterial cells, adherence, and phagocytosis of the bacteria are all initiated by binding of H. pylori SabA to sialic acid-carrying neutrophil receptors. This is the first time a distinct functional role has been defined for an H. pylori adhesin. The following events, linking receptor binding to oxidative burst reaction, involve a G-protein-linked signaling pathway and downstream activation of phosphatidylinositol 3-kinase, as shown by the inhibition experiments.

The SabA adhesin is present in ~40% of H. pylori strains and is subjected to phase variation (14). Individual H. pylori strains differ in their ability to bind to human neutrophils and to induce production of reactive oxygen radicals (11), and the reductions of the CL responses obtained by preincubation with sialylated oligosaccharides varies to some extent between different H. pylori strains (13). This might be due to a variable expression of the SabA adhesin caused by phase variation. Indeed, Western blot analysis using anti-SabA antibodies showed that the reference strain C-7050, which is devoid of neutrophil-activating capacity, does not express SabA (to be reported separately).

Rautelin et al. (8) showed that nonopsonized H. pylori cells phagocytosed by neutrophils resist phagocytic killing, in sharp contrast to those opsonized by serum complement. These observations are alike those described by Allen et al. who reported that type I H. pylori strains phagocytosed by bone marrow macrophages resist phagocytic killing, and after uptake the bacteria reside inside a novel form of vacuoles called megasomes (38). Furthermore, it was recently demonstrated that a novel phagocytic pathway regulated by atypical protein kinase C-ζ is involved in the uptake of H. pylori by bone marrow macrophages (39).

Interestingly, upon knock-out of the VacA cytotoxin, or the p37 fragment of VacA, in the sialic acid binding strain CCUG 17874 the initial extracellular burst was abrogated, while the second phase of the reaction was not affected. Thus, presumably, the p37 fragment of VacA contributes to the activation of the plasma membrane NADPH oxidase. This p37 fragment along with ~110 amino acids of the p58 part has been identified as the minimal intracellular vacuolating unit (40, 41). The p58 fragment, on the other hand, carries the cell binding capacity, but is devoid of vacuolating activity and is not internalized (23). The abrogation of the external burst obtained with the mutant strain expressing only the p58 fragment (17874/p37−) might indicate that the activation of the plasma membrane pool of the NADPH oxidase requires the channel-forming capacity, or (more likely) internalization of the toxin, and that binding of the toxin is not enough. These matters, however, need to be determined, and it should be noted that the effect observed was obtained with whole bacterial cells deleted of VacA or the p37 fragment and not with purified proteins.

Our results seem to be in contrast with the findings of Makristathis et al. (35), who reported that knock-out of VacA has no effect on neutrophil-activation caused by H. pylori. However, the flow cytometry method used in their study mainly detects intracellular oxygen radicals, and thus the effect on the external burst was not noted.

Deletion of the Lea-binding BabA adhesin did not affect the chemiluminescence response. Furthermore, no Lea-carrying
glycoconjugate has been identified in human neutrophils (42–45). Taken together this suggests that the Leb-binding capacity of \textit{H. pylori} is not involved in neutrophil activation.

The CL response obtained with the mutant deleted of the neutrophil-activating protein HP-NAP was also very similar to the response of the parental strain. This finding is in agreement with that of Leakey et al. (36) who showed that nonopsonic activation of neutrophils, as measured with luminol enhanced CL, was independent of HP-NAP. Correspondingly, no HP-NAP response was obtained with the present CL method when using pure protein (13). However, HP-NAP-induced production of reactive oxygen metabolites in human neutrophils may be measured with the monovanillic acid method (46) and the cytochrome c reduction method (47).

It is obvious from our findings and those of others that the demonstration of nonopsonic neutrophil activation by whole cells, sonicates, or soluble factors of \textit{H. pylori} is to some extent dependent on the methods used. Very few studies have, for example, compared whole cells with sonicates or purified proteins (13), and more studies are therefore needed to elucidate these conditions. Luminol-enhanced CL, used in the present study, offers in this respect some advantages, because it allows the demonstration of not only the external and internal oxidative bursts but also the study of their kinetics. Our findings thus showed that the immediate and rapid extracellular burst like that obtained with \textit{J99} wild type and NCTC 11637 is associated with rapid adherence of bacterial cells to the neutrophils. These initial events obviously activate the plasma membrane pool of NADPH oxidase. They are followed by phagocytosis for the next 20–30 min and activation of the pool of NADPH oxidase linked to neutrophil granules that is responsible for the internal burst (31). The rapid initial adherence and subsequent phagocytosis will apparently be responsible for the visible aggregation or agglutination of neutrophils by such strains that express SabA. Other strains like CCUG 17874, which also expresses SabA and activates neutrophils, may have a somewhat different kinetic pattern with a weaker external burst but a strong and prolonged internal burst. Of interest was the fact that the VacA of this strain seemed to play a role for the activation, because its VacA negative mutant abrogated the initial external burst but had no obvious influence on the internal. The reason for this is not clear but might have a clinical relevance, because Rautelin et al. (11) showed that VacA-producing strains with neutrophil-activating capacity were significantly more often found in patients with peptic ulcer disease than in those infected with strains lacking these phenotypic markers.

The signal transduction inhibitors DPI, pertussis toxin, and wortmannin inhibited or abrogated the nonopsonic neutrophil activation by whole \textit{H. pylori} cells. However, the adherence of \textit{H. pylori} to neutrophils was only moderately inhibited, indicating that the SabA still attached to the neutrophil receptor, whereas the signals were turned off. The same inhibitors used by us also inhibit the activation of human neutrophils induced by HP-NAP when measured by the monovanillic acid method (46). Thus, the signaling pathways employed by soluble HP-NAP and \textit{H. pylori} whole bacterial cells are very similar. Still, although the bacterial cells induce a strong CL response, no effect with HP-NAP was obtained in this assay. This might be due to the fact that very limited amounts of HP-NAP are present on the bacterial surface (48). It was recently shown that HP-NAP activates the extracellular regulated kinase and p38 mitogen-activated protein kinase in human neutrophils, and these events are essential for the HP-NAP-induced neutrophil respiratory burst and for the chemotaxis and adhesion of the neutrophils (47). Whether this is also the case with whole bacterial cells of \textit{H. pylori} needs to be determined.

Hansen et al. (49) have reported that the pertussis toxin does not inhibit the activation of neutrophils by \textit{H. pylori}. The reason for the discrepancy with our results presumably lies in the choice of methods for measuring. Hansen et al. studied the effects of sonicates of \textit{H. pylori}, whereas in this study whole bacterial cells were used. Furthermore, the concentration of luminol used by them was 70-fold higher than in our experimental system.

The term lectinophagocytosis was originally defined by studying the interactions between mannose-specific type-1 fimbriated bacteria and neutrophils (reviewed in Ref. 50). These bacteria also induce a neutrophil oxidative burst reaction, which may be inhibited by preincubation with mannose derivatives.

However, there are also nonmicrobial systems where protein-carbohydrate interactions lead to neutrophil activation. Thus, neutrophil oxidative burst may be induced by plant lectins, e.g., mannose-binding concanavalin A (51). Also carbohydrate-binding proteins present in mammalians, such as the \(\beta\)-galactose-binding galectin-1 and galectin-3, have the ability to activate the human neutrophil NADPH oxidase (52, 53).

The loss of \textit{H. pylori}-induced nonopsonic neutrophil activation upon deletion of the sialic acid-binding SabA adhesin shows that binding of the bacteria to sialic acid-carrying neutrophil receptors is required for induction of the oxidative burst and for phagocytosis. Interestingly, other sialic acid-binding ligands, such as influenza A virus and the lectin from the slug \textit{Limax flavus}, elicit a respiratory burst response in human neutrophils with formation of hydrogen peroxide, but with minimal accompanying superoxide generation (54, 55). The human influenza strain used in these studies, having an H3 hemagglutinin, preferentially recognizes glycoconjugates with terminal NeuAc6 residues, whereas the \textit{L. flavus} lectin binds to terminal NeuAc\textsubscript{6} irrespective of linkage position (57). The neutrophil activation was mediated via stimulation of phospholipase C and is not sensitive to pertussis toxin. This suggests that by binding to different sialic acid-carrying neutrophil receptors, influenza A virus and \textit{H. pylori} activates distinct signaling pathways leading to different types of neutrophil oxidative burst reactions.

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**Table III**

| Pretreatment of neutrophils with \(20 \text{ min} \) | Adherence/phagocytosis of \(H. pylori\) \(20 \text{–30 min} \) | Macroscopic neutrophil \(60 \text{–90 min} \) |
|--------------------------------------------------|-----------------------------------------------|---------------------------------|
| Diphenyleneiodonium chloride (DPI, 10 \(\mu\text{M} \) for 5 min) | +/− | | Not visible\(b\) |
| Pertussis toxin (800 \(\text{ng/ml for 120 min} \) | +/− | +/− | Not visible |
| Wortmannin (20 \(\text{nm} \) | +/− | +/− | Not visible |

\(a\) and \(+/−\) stand for observed minor and moderate adherence, respectively, whereas − stands for no observed phagocytosis.

\(b\) The test tubes were inspected under a stereomicroscope at a magnification of 15–20×.
In this study human neutrophil gangliosides were utilized to define sialic acid binding by *H. pylori*. However, some of the carbohydrate sequences of neutral gangliosides are also present on neutrophil glycoproteins (44), and sialic acid-dependent binding of certain *H. pylori* strains to glycoproteins of human neutrophils has also been demonstrated (Fig. 2) (21). Although the most likely receptor candidates are transmembrane glycoproteins, functional glycosphingolipid receptors for microbial proteins have also been described such as, e.g., the GM1 ganglioside receptor of cholera toxin (58, 59). Thus, so far it is an open question if the functional *H. pylori* neutrophil receptor is a glycosphingolipid or a glycoprotein.

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REFERENCES

1. Blaser, M. J. (1996) *Sci. Am.* 274, 104–107
2. Bahl, D., Anderson, A. K., O’Reilly, J., Fric, I. M., Kersulyte, D., Incecik, E. T., Berg, D. E., Covacci, A., Engstrand, L., and Borén, T. (1998) *Science* 279, 373–377
3. Stein, M., Rappuoli, R., and Covacci, A. (2001) in *Helicobacter pylori: Molecular and Cellular Biology* (Achtman, M., and Suerbaum, S., eds) pp. 227–244, Horizon Scientific Press, Norfolk, UK
4. Teneberg, S., and de Bernard, M. (2003) *Microbes Infect.* 5, 715–721
5. Szabo, I., Brutsche, S., Tombola, F., Moschioni, M., Satin, B., Telford, J. L., Rappuoli, R., Montecucco, C., Papini, E., and Zoratti, M. (1999) *EMBO J.* 18, 5517–5527
6. Tombola, F., Carlesso, C., Szabo, I., de Bernard, M., Reyrat, R. M., Telford, J. L., Rappuoli, R., Montecucco, C., Papini, E., and Zoratti, M. (1999) *Biophys. J.* 76, 1401–1409
7. Evans, D. J., Jr., Evans, D. G., Takemura, T., Nakano, H., Lampert, H. C., Graham, D. Y., Granger, D. N., and Kvietys, P. R. (1995) *Infect. Immun.* 63, 2213–2220
8. Rautelin, H., Blomberg, B., Fredlund, H., Jarnerot, G., and Danielsson, D. (1995) *Gut* 34, 599–603
9. Satk, S. C., Youn, H. S., Chung, M. H., Lee, W. K., Cho, M. J., Ko, G. H., Park, S. J., and Kim, S. H. (1996) *Cancer Res.* 56, 1279–1282
10. Yoshikawa, T., and Naito, Y. (2000) *Curr. Biol.* 10, 787–794
11. Bylund, J., Christophe, T., Boulay, F., Nystrom, T., Karlsson, A., and Dahlgren, C. (2001) *Antimicrob. Agents Chemother.* 45, 1700–1704
12. Allen, L.-A. H., Schlesinger, L. S., and Blakely, S. R. (1999) *J. Biol. Chem.* 274, 9277–9282
13. Maier, B. A., and Lack, C. J. (1980) *J. Biol. Chem.* 255, 2092–2096
14. Sponer, E., Fukuda, M., Klock, C. J., Oates, J. E., and Dell, A. (1984) *J. Biol. Chem.* 259, 4782–4801
15. Ye, D., Willhite, D. C., and Blakely, S. R. (1999) *J. Biol. Chem.* 274, 115–127
16. Allen, L.-A. and Allgood, J. A. (2002) *Curr. Biol.* 12, 1762–1766
17. de Bernard, M., Burrini, D., Papini, E., Rappuoli, R., Telford, J., and Montecucco, C. (1998) *Infect. Immun.* 66, 6014–6016
18. Ve, D., Willhite, D. C., and Blakely, S. R. (1999) *J. Biol. Chem.* 274, 9277–9282
19. Sponer, E., Fukuda, M., Klock, C. J., Oates, J. E., and Dell, A. (1984) *J. Biol. Chem.* 259, 4782–4801
20. Satin, B., Del Guidice, G., Della Bianca, V., D. S., Laudanna, C., Tonello, F., Kelleher, D., Rappuoli, R., Montecucco, C., and Rossi, F. (2000) *J. Exp. Med.* 19, 1467–1476
21. Ishihara, H., Baesso, I., Sanenziato, G., Trentin, L., Rappuoli, R., Dell Guidice, G., and Montecucco, C. (2003) *Eur. J. Immunol.* 33, 840–849
22. Blom, K., Lundin, S., Bolin, D., and Svennerholm, A.-M. (2001) *FEBS Immunol. Med. Microbiol.* 30, 173–179
23. Hansen, P. S., Madsen, P. H., Petersen, S. B., and Nielsen, H. (2001) *Clin. Immunol.* 123, 73–80
24. Okf, I., and Sharon, N. (1988) *Infect. Immun.* 56, 539–547
25. Cohen, M. S., Metzcal, J. A., and Root, R. K. (1980) *Blood* 55, 1003–1010
26. Karlsson, A., Pollin, P., Leffier, H., and Dahlgren, C. (1998) *Blood* 91, 3430–3438
27. Almkvist, J., Dahlgren, C., Leffler, H., and Karlsson, A. (2002) *J. Immunol.* 169, 4034–4041
28. Hartshorn, K. L., Collamer, M. W., White, M. R., Schwartz, J. H., and Tauber, A. I. (1990) *Blood* 75, 218–226
29. Hartshorn, K. L., Daignault, D. E., White, M. R., and Tauber, A. I. (1992) *J. Leukoc. Biol.* 51, 239–236
30. Connor, R. J., Kawaoka, Y., Webster, R. B., and Paulson, J. C. (1994) *Virology* 205, 17–23
31. Knibbs, R. N., Osborne, S. E., Glick, G. D., and Goldman, I. J. (1993) *J. Biol. Chem.* 268, 18524–18531
32. Polberg, J., Linnroth, I., and Svennerholm, L. (1973) *Infect. Immun.* 8, 208–214
33. Pavszczak, T., and Fishman, P. H. (1990) *J. Biol. Chem.* 265, 7673–7678
34. UPEC-IB Joint Commission on Biochemical Nomenclature (JCBN) (1998) *Eur. J. Biochem.* 259, 293–298