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Characterization of a *Helicobacter pylori* Neutrophil-Activating Protein

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***Helicobacter pylori*-associated gastritis is mainly an inflammatory cell response. In earlier work we showed that activation of human neutrophils by a cell-free water extract of *H. pylori* is characterized by increased expression of neutrophil CD11b/CD18 and increased adhesiveness to endothelial cells. The work reported here indicates that the neutrophil-activating factor is a 150,000-molecular-weight protein (150K protein). Neutrophil proadhesive activity copurified with this protein, which is a polymer of identical 15K subunits. Specific antibody, prepared against the purified 15K subunit, neutralized the proadhesive activity of the pure protein and of water extracts obtained from different strains of *H. pylori*. The gene (*napA*) for this protein (termed HP-NAP, for *H. pylori* neutrophil-activating protein) was detected, by PCR amplification, in all of the *H. pylori* isolates tested; however, there was considerable strain variation in the level of expression of HP-NAP activity in vitro. HP-NAP could play an important role in the gastric inflammatory response to *H. pylori* infection.**

Helicobacter pylori is closely associated with gastric and peptic ulcer disease and with the development of duodenal ulcers (9, 21, 22, 39, 51). Chronic *H. pylori* gastritis is also a major risk factor for development of gastric cancer (8, 27, 44, 54). *H. pylori* infection elicits an inflammatory cell response, and the severity of mucosal injury appears to be directly correlated with the extent of neutrophil infiltration (9, 11, 13, 14, 18, 22, 32, 37, 38). It has been suggested that activated leukocytes may be responsible for some of the tissue damage seen in cases of *H. pylori* gastritis (5, 13, 14, 22, 35, 42) and that activated phagocytes, especially in the presence of *H. pylori* products, contribute to carcinogenesis (53, 55).

Since the primary consequence of *H. pylori* infection is gastritis, many investigations have centered on the mechanisms involved in the gastric inflammatory response (10, 11, 13, 14, 18, 25, 32, 34, 37, 38, 40, 46, 47, 52, 57). Cell-bound components of *H. pylori*, artificially released by either sonication or extraction, include factors which are chemotactic for neutrophils and monocytes (11, 34, 37, 40, 47). One study showed that the N-terminal end of the large subunit of *H. pylori* urease was chemotactic for neutrophils (37). Other, as yet unidentified, soluble chemotactic factors of *H. pylori* have also been described (11, 34, 47). We recently reported that water extracts of *H. pylori* contain one or more factors which increase surface expression of the CD11b/CD18 on neutrophils and increase adhesion of neutrophils to endothelial cells in vivo and in vitro (57). We also showed that *H. pylori*-induced neutrophil adhesion to endothelial cells is a CD11b/CD18–ICAM-1-dependent event (57). The proadhesive activity of the *H. pylori* extract could not be attributed to molecular mimics of leukotriene B_4, platelet-activating factor, or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (57). Preliminary characterization of the proadhesive factor of *H. pylori* 8826 indicated protein-like properties (i.e., heat lability and pronase sensitivity but acid and pepsin resistances) (57). Here, we describe experiments which identify one *H. pylori* neutrophil-activating factor as a 150,000-molecular-weight multimeric protein (150K protein) which we purified from water extracts of *H. pylori.*

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MATERIALS AND METHODS

Bacteria and growth conditions. *H. pylori* 8826 (MG26, according to the terminology used in this report; see below) and other test strains were isolated from antral stomach biopsies (15, 23, 24). Of the 21 isolates, 15 were obtained from men, including 8 isolates obtained from men with duodenal ulcers (designated by the prefix DU), 1 isolate obtained from a man with a gastric ulcer (prefix, GU), and 6 isolates obtained from men with gastritis (prefix, MG); 6 isolates were obtained from women with gastritis (prefix, FG). Ages (in years) ranged from 23 to 78, and the mean age for the group was 47.

The test strains were stored (long term) as a thick cell suspension in *Campylobacter* Albimi Cysteine medium (Remel, Lexana, Kans.) containing 20% glycerol (vol/vol), at −70°C. For extraction, *H. pylori* was grown on blood agar consisting of brain heart infusion (Difco Laboratories, Detroit, Mich.), 0.5% Bacto yeast extract (Difco), 2.0% agar (Difco), and 7% fresh horse blood; the strains were incubated for 36 to 48 h at 37°C in an atmosphere of 12% CO_2 in air and 98 to 100% humidity. The cyclodextrin-based agar medium described by Olivieri et al. (42), prepared with 0.1% beta-cyclodextrin (Sigma Biochemical Co., St. Louis, Mo.) instead of 0.1% methyl-beta-cyclodextrin, was used to grow *H. pylori* in the absence of blood products.

***H. pylori* water extracts.** *H. pylori* cells were extracted by harvesting into distilled water, 1.0 ml for each plate, and by centrifugation (17,000 × *g*, 15 min) after keeping the suspension at room temperature for 20 min. All of the crude extracts employed for the adhesion assays consisted of this initial supernatant, which consistently contained 15 to 18 mg of protein per ml even when different strains were compared. The extracts employed for protein purification were water extracts subsequently obtained by resuspending the first cell pellet in distilled water, using two-thirds of the original volume, keeping the suspension at room temperature for 40 min, and centrifugation at 25,000 × *g* for 15 min. The protein composition and proadhesive activity of this second water extract were essentially the same as those of the first water extract. No preservatives were added; the extracts were stored at −20°C until needed. Prior to use, the extracts were rapidly thawed and centrifuged at high speed (39,000 × *g*) for 20 min, the pellet was discarded, and the supernatant fluid was submitted to filtration with a 0.2-μm-pore-size Acrodisc filter (Gelman Sciences, Ann Arbor, Mich.). This procedure was carried out to remove a large proportion of the very-high-molecular-weight bacterial components such as lipopolysaccharide (LPS)-containing membrane vesicles and intact flagella.

Protein purification. Initial fractionation of the water extract was by A-1.5m agarose (Bio-Rad Laboratories, Richmond, Calif.). chromatography, using a Pharmacia (Uppsala, Sweden) column (1.6 by 100 cm), applying 6.4-ml samples, and eluting 2.5-ml fractions. The elution buffer was 0.1 M sodium phosphate buffer, pH 7.2, in phosphate-buffered saline (PBS) or, for protein purification, 0.05 M Tris-Cl buffer, pH 8.0; both types of elution buffer gave the same elution profile. Fractions containing proteins in the molecular-weight range of 50,000 to

TABLE 1. Copurification of neutrophil proadhesive activity with a 150K *H. pylori* protein from water extracts

Sample source[a]	Vol (ml)	Protein (mg/ml)	Titer[b]	Protein (mg/ml at titer)	Units/ml[c]	Total units	Total protein
Extract	160	15.50	1:350	0.00886	112.9	18,059	2,480
Agarose	225	1.00	1:75	0.01333	75.0	16,875	225
Superdex	72	0.80	1:180	0.00444	225.2	16,216	58
MonoQ	9	0.11	1:160	0.00069	1,449.3	13,044	1

[a] Samples were taken from crude water extract and following passage through agarose, Superdex, and MonoQ columns.
[b] Titer, highest dilution producing proadhesive activity equal to twice the baseline value in the HUVEC-neutrophil adhesion assay.
[c] Units, reciprocal of milligrams of protein per milliliter at the titer value; units/ml, specific activity.

200,000, which correlated with a peak of proadhesive activity (57), were pooled and concentrated to 0.1 volume, using Centricon-10 microconcentrator devices (Amicon, Inc., Beverly, Mass.). Samples, 5.6 ml each, were eluted through a Pharmacia Superdex-200 column by using the Pharmacia-LKB fast protein liquid chromatography system and by collecting 3.0-ml fractions with 0.025 M Tris-Cl buffer, pH 8.0, containing 0.05 M NaCl. The most active fractions were again pooled and concentrated approximately fivefold, as described above, and applied to a Pharmacia MonoQ ion-exchange column. Only one major protein, with a molecular weight of approximately 150,000, was not retained by this column.

In a typical purification scheme, the starting material was four batches of crude second water extract, with a total volume of 160 ml, prepared as described above, concentrated to 32 ml, and passed through the agarose column in five runs. Eighteen peak fractions, with a total volume of 225 ml, were pooled and concentrated 10-fold as described above, and four 5.6-ml samples were passaged through the Superdex column. The six 3.0-ml samples containing the 150K protein peak, with a total volume of 72 ml, were pooled and concentrated approximately fivefold. The entire sample was eluted though the MonoQ column, and all of the eluate was collected and reconcentrated to a total volume of 9.0 ml. Optical density was monitored at 280 nm to avoid overloading the MonoQ column.

SDS-PAGE analysis and Western blotting (immunoblotting). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (36) was performed with 12% gels and 40 to 60 μg of protein per lane. Protein bands were stained with Coomassie blue.

Antiserum against the 15K subunit of the 150K protein was obtained by immunizing a rabbit with MonoQ column-purified protein electroeluted from an SDS-polyacrylamide gel. Standard Western immunoblotting was performed after electrotransfer to nitrocellulose paper. Reagents included anti-15K subunit rabbit serum diluted 1:1,000 in PBS plus 0.02% Tween 20, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma Biochemical Co.), and nitroblue tetrazolium (NBT) plus 5-bromo-4-chloro-3-indolylphosphate as the substrate.

Assay for the *H. pylori* neutrophil-activating protein (HP-NAP) gene, *napA*, by PCR amplification of a 344-bp internal DNA segment. Template DNA was prepared by boiling, for 5 min, a freshly prepared bacterial suspension obtained by harvesting the growth from one blood agar plate into 500 μl of sterile water, using a sterile disposable inoculating loop. Two microliters of template DNA was added to a 100-μl reaction mixture consisting of 10 mM Tris-Cl buffer, pH 8.3, containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 2.5 U of *Taq* polymerase, and 0.2 μM each primer, overlaid with 60 μl of mineral oil. Oligonucleotide primers Nap-1 (19-mer, 5′-TGCGATCGTGTTG TTTATG-3′) and Nap-2 (19-mer, 5′-GATCGTCCGCATAAGTTAC-3′) were synthesized at the Molecular Genetics Core Facility of Baylor College of Medicine, using a Model 394 automated DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.), and purified in G-25 (Pharmacia) columns. PCR was performed with an automatic thermal cycler (Perkin-Elmer Cetus), as follows. The first, denaturation, cycle was for 5 min at 94°C; this was followed by 25 cycles of denaturation at 94°C for 1 min, primer annealing at 46°C for 2 min, and DNA chain extension at 72°C for 2 min. The final five cycles were for 8 additional min at 72°C, followed by rapid cooling to 4°C. Each experiment included a negative control with no DNA template added. Amplified products were analyzed by electrophoresis in a 2% agarose gel, by standard procedures.

The 344-bp PCR product was confirmed by cloning and sequencing, as follows. *Escherichia coli* host strain INV*alpha* (Invitrogen Corp., San Diego, Calif.) was used with the PCR-II™ vector (Invitrogen), using standard methods (48). Single-stranded DNA was prepared from selected clones by the polyethylene glycol method (49) with the forward M13 universal primer and a Model 373A DNA sequencer (Applied Biosystems), as recommended by the manufacturer.

Neutrophil-HUVEC adhesion assay. The procedures used to obtain human endothelial cells and neutrophils were approved by the Institutional Review Board for Human Research at the Louisiana State University Medical Center. All subjects provided written consent and were reimbursed for their time.

Human umbilical vein endothelial cells (HUVEC) were obtained from umbilical cords by collagenase treatment as previously described (56). HUVEC were grown in Medium 199 (GIBCO, Grand Island, N.Y.) plus supplements and antibiotics and identified, as recently described (57). HUVEC were incubated at 37°C in a humidified atmosphere with 5% CO_2 and expanded by brief trypsinization (0.25% trypsin in PBS containing 0.02% EDTA). Primary- through third-passage HUVEC were seeded into gelatin (0.1%)- and fibronectin (25 μg/ml)-coated 11-mm, 48-well tissue culture plates (GIBCO) and used for adhesion assays when confluent. Human polymorphonuclear leukocytes (PMN) were obtained from healthy adults and isolated as previously described (57). The PMN population was 95 to 98% viable (by trypan blue exclusion) and 98% pure (by acetic acid-crystal violet staining).

For adhesion assays, isolated neutrophils were suspended in PBS and radiolabeled by incubating the cells at 2×10^7 cells per ml with 30 μCi of $Na^{51}CrO_4$ per ml of neutrophil suspension at 37°C for 60 min. The cells were washed twice with cold PBS at $250 \times g$ for 8 min to remove unincorporated radioactivity and then suspended in plasma-free Hanks balanced salt solution. Labeled neutrophils were added to HUVEC monolayers at a neutrophil-to-endothelial cell ratio

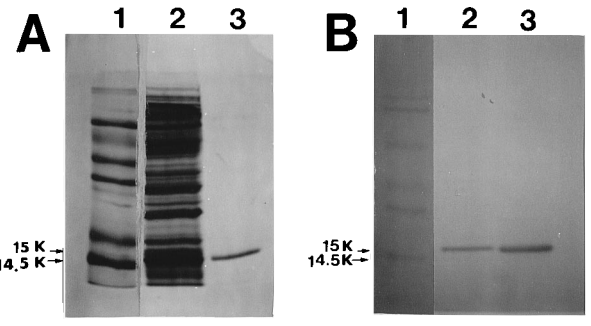

FIG. 1. Purification of the *H. pylori* 150K protein from crude water extract. SDS-PAGE results showing the 15K subunit in crude extract (lanes 2) and after passage through a MonoQ column (lanes 3); the position of the 15K subunit is compared with that of a 14.5K molecular weight standard in lanes 1. (A) Gel stained with Coomassie blue. (B) Western immunoblot developed with rabbit anti-15K subunit antibody.

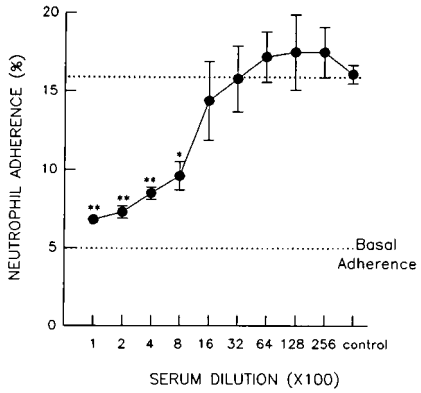

FIG. 2. Neutralization of 150K protein-enhanced neutrophil adherence to endothelial cells by serial dilutions of rabbit anti-15K subunit antibody. Basal adherence was measured with no 150K protein added; control adherence (top dotted line) was measured with no antibody added and with equivalent dilutions of normal rabbit serum. The final concentration of *H. pylori* MonoQ-purified 150K protein was 0.02 mg/ml. *, $P < 0.01$; **, $P < 0.03$, versus control.

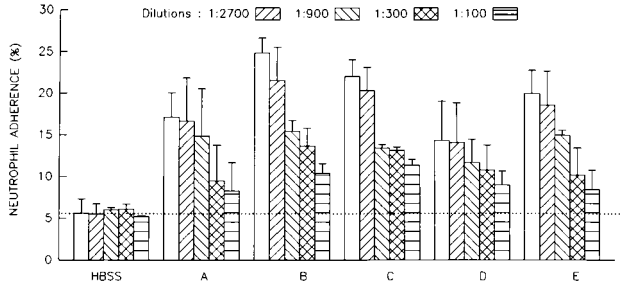

FIG. 3. Neutralization of *H. pylori* (water extract)-enhanced neutrophil adherence to endothelial cells by serial dilutions of rabbit anti-150K protein antibody. Water extracts were diluted 1:10. Open bars, no rabbit anti-15K subunit antibody. Basal adherence (dotted line) was measured with no water extract added. HBSS (Hanks balanced salt solution), diluent with or without antibody, as indicated by the bars. Water extracts were as follows: primary (A) and secondary (B) extracts of *H. pylori* MG26 grown on synthetic, cyclodextrin-based agar medium; primary (C) and secondary (D) extracts of strain MG25 grown on blood-based agar medium; and primary extract (E) of strain FG146 grown on blood-based agar medium.

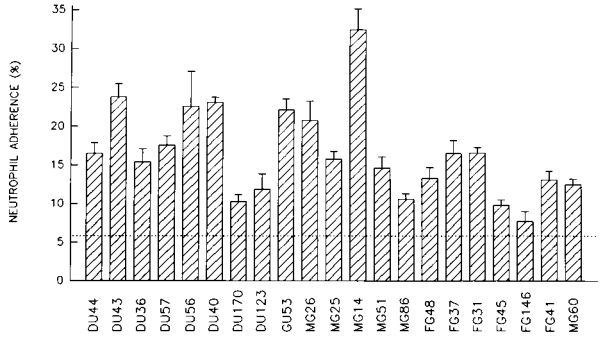

FIG. 4. Enhancement of neutrophil adherence to endothelial cells by water extracts of 21 different strains of *H. pylori*. Basal adherence (dotted line) was measured with no water extract added; the final dilution of extracts was 1:40. The strain designations are defined in Materials and Methods. Means ± standard errors are shown.

of 10:1 with or without *H. pylori*-derived samples. After coincubation (30 min), the percent of added neutrophils that remained adherent to the HUVEC monolayers was quantitated, as follows (57). The supernatant was removed, the monolayers were washed, and the remaining cells were lysed; percent adherent neutrophils was calculated by dividing [lysate cpm (^{51}Cr activity) × 100] by [supernatant cpm + wash cpm + lysate cpm].

Assay of antibody neutralization of *H. pylori*-induced neutrophil adhesion to HUVEC was performed by combining one-half the indicated dilution of rabbit antiserum (anti-150K protein antibody) with an equal volume of 2× neutrophil suspension plus *H. pylori* extract. After preincubating the mixture for 60 min at 37°C, an appropriate aliquot was added to HUVEC monolayers and adhesion was assessed 30 min later.

NBT dye reduction assay. PMN were obtained as noted above (57). For the NBT dye reduction assay (29), 1.0-ml reaction mixtures consisted of 1×10^6 PMN suspended in minimal essential medium supplemented with calcium (GIBCO) plus 0.02% NBT; additions included none (negative control), 10 or 20 nM fMLP (Sigma) (positive control), or 1.1 or 3.3 μg of purified HP-NAP. After incubation at 37°C for 30 min, the reaction was stopped by incubation in ice for 5 min and duplicate cytospins (Shandon Cytospin 2; Shandon, Inc., Pittsburgh, Pa.) prepared after diluting each mixture 1:5. After being cytospun, the cells were fixed with chilled ethanol and counterstained with Gram's safranin (Difco). The cells were then observed microscopically for presence of the formazan precipitate. Results were expressed as the percentage of cells containing heavy formazan precipitate.

Statistical analysis. Data were analyzed by analysis of variance and Student's *t* test (with Bonferroni corrections for multiple comparisons). Data are expressed as means ± standard errors.

RESULTS

Purification of a 150K protein from water extracts of *H. pylori*. As previously reported (57), fractionation of *H. pylori* water extracts by agarose chromatography indicated that at least one active component was a protein of high molecular weight but smaller than *H. pylori* urease. SDS-PAGE analysis of the agarose column fractions revealed an apparent correlation between presence of a multimeric 150K protein, having a 15K subunit, and neutrophil-activating activity. Further purification of this protein by molecular sieving was achieved by Superdex-200 column chromatography with a buffer containing 0.05 M NaCl, producing a better-defined protein peak having proadhesive activity. The protein of interest eluted in exactly the same position as the 150K yeast alcohol dehydrogenase used as a molecular weight standard (not shown), confirming the previous molecular weight estimate.

Final purification of the 150K protein was achieved with a MonoQ ion-exchange column, exploiting the fact that this was the only protein not retained by the column in the presence of 0.05 M NaCl. Table 1 shows that the proadhesive activity purifies along with the 150K protein, with the purified protein active at about 0.7 μg/ml. Activity lost in the agarose column step includes that which eluted in the void volume of the column. Figure 1 compares crude water extract and purified 150K protein, showing the 15K subunit, following SDS-PAGE, on a gel stained with Coomassie blue (Fig. 1A) and an immunoblot developed with anti-15K subunit antibody-conjugate (Fig. 1B).

Neutralization of *H. pylori* proadhesive activity by specific antibody. Figure 2 shows the results obtained when rabbit anti-15K subunit antibody was titrated for neutralization of neutrophil adherence enhanced by the purified native protein. Approximately 85% neutralization was achieved with serum diluted 1:100, and significant neutralization can be seen at a serum dilution of 1:800. Controls consisting of normal rabbit serum and rabbit preimmune serum showed no neutralization of proadhesive activity at any dilution.

Figure 3 shows antibody neutralization of the proadhesive activity in primary and secondary water extracts of *H. pylori*, grown on blood agar plates and on a synthetic medium which does not contain blood or serum. These results show that there is essentially no difference between the active factor obtained in the primary and secondary water extracts and that the active factor is not unique to strain MG26. Also, proadhesive activity in *H. pylori* water extracts is independent of the presence of whole blood or serum in the growth medium. This eliminates the possibility that a component of horse blood might be involved in the proadhesive activity of the *H. pylori* water extracts.

Proadhesive activity of water extracts of various *H. pylori* strains and neutralization by anti-15K subunit antibody. Assay of the proadhesive activity of 21 different isolates of *H. pylori* showed that water extracts of all strains tested have this activity, although there is obvious variation in the intensity of proadhesive activity. Eight of the extracts produced only a 50 to 100% increase in neutrophil adherence, seven extracts produced a 100 to 200% increase, five extracts produced a 200 to 300% increase, and one extract produced a 400% increase above the baseline (Fig. 4). This variation was not due to differences in protein concentrations in the extracts.

Western immunoblots of the extracts showed that all of the test strains produced a 15K subunit protein reactive with the antibody prepared against the protein of strain MG26 (Fig. 5). Some strains appeared to be relatively free of cross-reactive proteins (Fig. 5A), while others showed one or more other bands reactive with the anti-15K subunit antibody (Fig. 5B); some of these bands could be multimers of the 15K subunit,

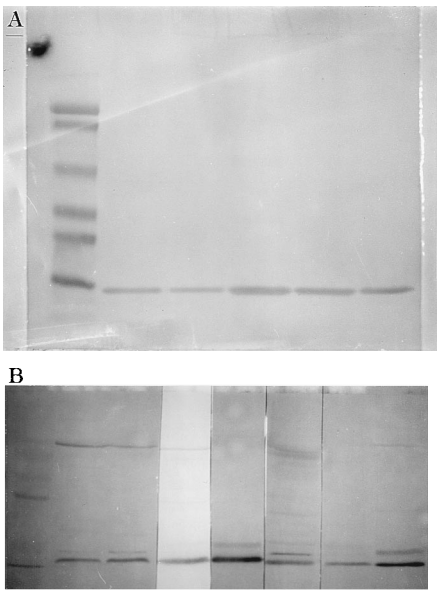

FIG. 5. SDS-PAGE analysis of water extracts of *H. pylori* strains for 15K subunit of the 150K protein. (A) Extracts of five different strains (left to right [beginning with lane 2]: strains MG26, MG25, FG31, DU43, and DU36) showing only the 15K subunit protein band. (B) Composite showing extracts of six strains showing the 15K subunit protein band plus other antibody-reactive protein bands (left to right [beginning with lane 2]: DU36, FG48, DU56, MG60, DU123, MG26, and FG37). In panel A, lane 1 contains Bio-Rad low-molecular-weight prestained standards. In panel B, lane 1 contains Bio-Rad kaleidoscope prestained standards as follows: 202K, 133K, 71K, 41.8K, 30.6K, 17.8K and 6.9K. The 17.8K standard appears to be 16K in this preparation; note that samples in panel A, lane 2, and in panel B, lane 7, are the same.

i.e., incompletely dissociated 150K protein. There was no apparent correlation between the appearance of cross-reactive protein bands and the relative level of proadhesive activity. There was also no apparent correlation between the amount of proadhesive activity and the source of the isolates; i.e., gastritis with or without duodenal ulcer.

Figure 6 shows the results of an experiment in which the proadhesive activity of primary water extracts of nine different *H. pylori* isolates was assayed in the presence and in the absence of the anti-15K subunit serum. As can be seen, the degree of neutralization of the proadhesive activity also showed strain variation. Although we suspect interference by

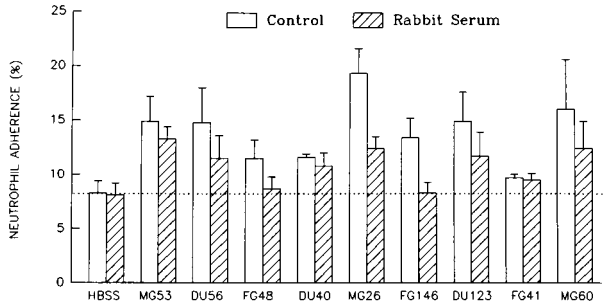

FIG. 6. Neutralization of *H. pylori* (water extract)-enhanced neutrophil adherence to endothelial cells by rabbit anti-150K protein antibody: *H. pylori* strain variation. Water extracts from nine different strains were assayed at a dilution of 1:40, with or without (control) anti-15K subunit antibody added at a final dilution of 1:200. Basal adherence (dotted line) was measured with no water extract added. HBSS (Hanks balanced salt solution), diluent with or without antibody, as indicated by the bars. Means ± standard errors are shown.

TABLE 2. Activation of neutrophils by fMLP and HP-NAP demonstrated by reduction of NBT

Addition	% Activated[a]	
	Expt 1	Expt 2
10 nM fMLP	77.6	72.7
20 nM fMLP	90.0	92.0
1.1 μg of HP-NAP	62.5	70.0
3.3 μg of HP-NAP	90.5	84.9
None (with NBT)	0.0	5.0
None (without NBT)	0.0	0.0

[a] One hundred cells were counted, in duplicate in each experiment; activated neutrophils were those containing a heavy formazan precipitate.

other components of the extracts, the basis of this variation is unclear. Another possibility is that self-aggregation of the 150K protein during storage might affect the ability of the protein to activate neutrophils and/or its susceptibility to antibody neutralization.

Stimulation of reactive oxygen radical production in neutrophils by HP-NAP. Upregulation of neutrophil CD11b/CD18 is accompanied by an increase in membrane NADPH-oxidase activity, resulting in generation of reactive oxygen radicals (1, 3, 4, 12, 25, 29). We used the NBT dye reduction assay (29), in which NBT is reduced by reactive oxygen radicals to a visible intracellular formazan precipitate, to test for this response in neutrophils activated by HP-NAP. Table 2 shows the results obtained when neutrophils were exposed to fMLP or purified HP-NAP in the presence of NBT. It can be seen that both fMLP and HP-NAP stimulated reactive oxygen radical production in a dose-dependent manner. Figure 7 shows representative results; note that neutrophils exposed to NBT alone show only a few formazan grains per cell, while those exposed to NBT in the presence of fMLP or HP-NAP contain large formazan precipitates.

Detection of the gene, *napA*, encoding HP-NAP in test strains by PCR amplification of an internal gene sequence. A 68-residue N-terminal amino acid sequence was obtained for the 15K subunit of HP-NAP; this allowed us to determine the nucleotide sequence for the entire gene in strain MG26, which has been reported elsewhere (reference 16; GenBank accession number, U16121). The size and amino acid sequence of HP-NAP identify it as belonging to the bacterioferritin family of proteins (2, 6, 16, 50), similar but not identical to the procaryotic ferritin of *H. pylori* reported by others (16, 19); however, this does not preclude possession of a receptor-binding amino acid motif, which is typically tripeptide in size (30, 43, 45, 54), responsible for interaction with neutrophils (see Discussion).

As detailed in Materials and Methods, synthetic oligonucleotide primers were used to PCR amplify a 344-bp internal sequence of *napA*. DNA from all of the test strains was used to test for the presence of this *napA*-specific sequence, and all were found to be positive. Figure 8 shows the results obtained with five of the strains.

DISCUSSION

H. pylori gastritis is characterized by a protracted inflammatory cell response, abnormal gastric physiology, and gastric mucosal cell injury. Numerous investigations have shown that treatments which include agents which eliminate *H. pylori* from the stomach resolve gastritis and cure duodenal ulcer lesions (23, 24, 31). There is considerable clinical and experimental evidence that *H. pylori* attracts inflammatory cells to the site of

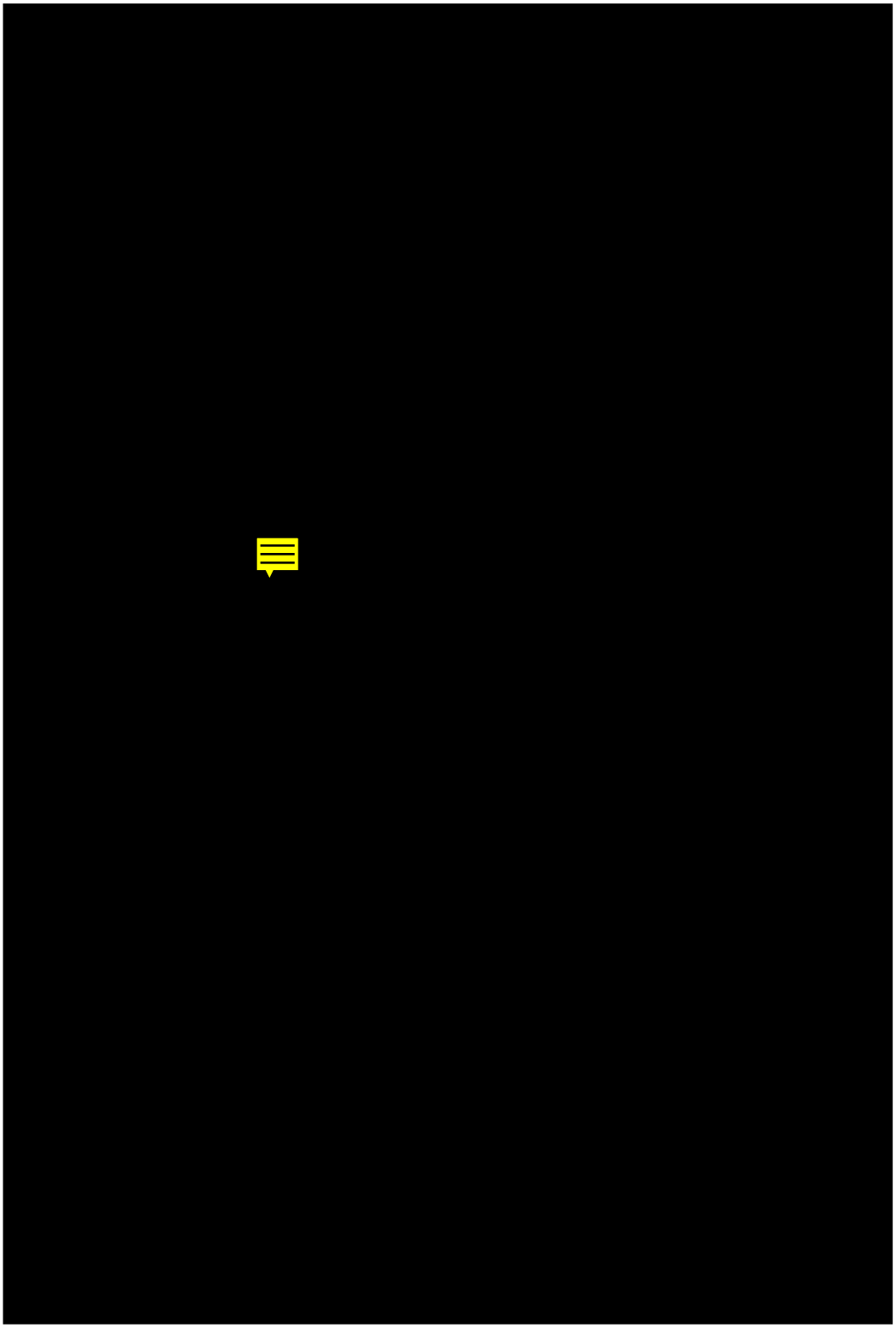

FIG. 7. Production of reactive oxygen radicals, determined by reduction of NBT dye to formazan, by neutrophils activated with either fMLP or HP-NAP. Neutrophils were unstimulated (A), unstimulated, NBT control (B), stimulated with 20 nM fMLP (C), and stimulated with 3.3 μg of purified HP-NAP (D). For panels B through D, NBT was in the reaction mixture. Note the large deposits of formazan precipitates within the neutrophils in panels C and D.

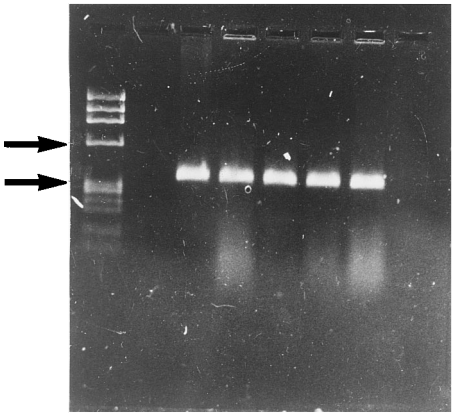

FIG. 8. Assay for *napA* gene in different strains of *H. pylori* by PCR amplification of a 344-bp internal fragment defined by *napA*-specific primers. Lanes (from left to right): 1, ϕX174 *Hae*III DNA digest standards 1,353, 1,078, 872, 603, 310, 281, 234, 194, 125, and 72 bp [arrows indicate the 603- and 310-bp fragments]; 2, negative control with no template DNA added; 3 through 7, DNA from *H. pylori* DU123, MG60, DU36, MG53, and DU56, respectively.

infection and that reactive oxygen metabolites released by phagocytes activated by *H. pylori* cause local mucosal damage (13, 14) but *H. pylori* somehow escapes the lethal effects of this cellular response. Davies et al. (13) have recently confirmed that there is a highly significant correlation between *H. pylori* burden, histologically assessed gastric mucosal damage, and mucosal reactive oxygen metabolite production. An understanding of the exact molecular mechanism(s) by which *H. pylori* induces a chronic inflammatory response and its resultant gastric mucosal injury should lead to improved approaches for preventing and curing *H. pylori* gastritis.

We and other workers have demonstrated that *H. pylori* activates monocytes and PMN (neutrophils); this activity has been associated with cell-bound factors, with cell-free factors naturally released into broth culture fluids, and also with factors released by sonication or extraction (11, 34, 37, 40, 47, 57). *H. pylori* activation of neutrophils is evident as a chemotactic response, as an oxidative burst response, and as an increase in adhesion to endothelial cells (41, 46, 57). To date, there has been little or no consensus concerning the molecular nature of *H. pylori* chemoattractants/neutrophil activators, except that the factor(s) probably does not include either LPS or fMLP-like peptides (34, 38). The results of our previous work (57) and that presented here also indicate that *H. pylori* activation of human neutrophils does not involve either LPS, fMLP, LTB_4, or platelet-activating factor.

Use of the neutrophil-HUVEC adhesion assay in the work reported here was based on our original observation that activation of neutrophils by a water extract of *H. pylori*, quantitated by this assay, involves upregulation of CD11b/CD18 integrin (57). However, it should be noted that CD11b/CD18 upregulation also results in increased iC3b binding, increased chemotaxis, increased transendothelial migration, and increased phagocytosis and oxidative burst response (3, 4, 12, 57). Also, stimulation of neutrophils with chemotactic agents leads to upregulation of CD11b/CD18 (28, 33).

In activated neutrophils, reactive oxygen radicals are produced as the result of activation of membrane NADPH-oxidase (29). Several different methods have been designed for quantitating reactive oxygen radical production, including cytochrome *c* reduction, luminol- and lucigenin-enhanced chemiluminescence, and NBT dye reduction (1, 25, 29). To further characterize the effect of HP-NAP on neutrophils, we measured NBT reduction by neutrophils exposed to purified HP-NAP or fMLP. We found that, like fMLP, HP-NAP stimulates NBT reduction, which is consistent with CD11b/CD18 upregulation and increased adhesion to endothelial cells.

The studies reported here concern identification and partial characterization of an *H. pylori* protein which promotes neutrophil adhesion to the endothelium. Our previously reported observation (57) that the proadhesive activity was associated with very-high (10^6)-, medium (10^5)-, and also relatively low-molecular-weight (10^4) material is entirely consistent with the properties of HP-NAP; i.e., 15K monomers forming a 150K molecule which can form higher-molecular-weight aggregates. These properties are also consistent with identification of HP-NAP as a bacterioferritin-type protein (16). Using a specific antiserum to perform immunoblots, we have confirmed that a proportion of the 150K protein elutes in and near the void column of agarose columns, presumably in the form of very-high-molecular-weight aggregates, and that the 15K subunit of this protein is present in the low-molecular-weight fractions, which also have significant proadhesive activity.

Although we identified HP-NAP as a bacterioferritin on the basis of its amino acid sequence, subunit structure, and conservation of a ferroxidase center motif (16), the metabolic function of this protein in *H. pylori* is not clear, especially since the organism is known to have another bacterioferritin with proven iron storage function (2, 19) and we have seen no evidence of iron or heme content in HP-NAP. It is interesting that the protein antigens 4D of *Treponema pallidum* and antigen D of *Mycobacterium paratuberculosis* have proven to be bacterioferritins (6, 16, 17).

The fact that antibody prepared against the subunit protein, purified from strain MG26, failed to neutralize part of the proadhesive activity in water extracts of some of the test strains is under investigation. One possibility is that antibody against the 15K subunit is not ideal for this purpose; this is supported by the fact that the activity of the 150K protein was not 100% neutralized by this antibody. Another obvious possibility is that some strains also produce an antigenically unrelated NAP(s). PCR amplification of an internal sequence of the HP-NAP gene, *napA*, confirmed that all of the strains tested possess the gene, and the presence of HP-NAP was demonstrated, by immunoblot, in crude extracts with the anti-15K subunit antibody. Protein bands of various molecular weights and reactive with the anti-15K subunit antibody were also detected in many *H. pylori* strains. Some of these cross-reactive bands may actually be multimers of the 15K protein, and others may be functionally unrelated proteins which could interfere with antibody neutralization of the proadhesive activity of the 150K protein. We also suspect that crude extracts from different strains might contain different ratios of monomer, 150K, and higher-molecular-weight aggregates, which could affect both the amount of activity detected and the ability of the anti-subunit antibody to neutralize the proadhesive activity. This idea is supported by the observation that the activity in crude extracts of some other strains appeared to show greater neutralization than that of strain MG26. It should be noted that the activity, neutralization, and immunoblot assays performed for this phase of work were not designed to answer all of these questions.

The molecular basis of the proadhesive activity of HP-NAP remains unknown, but presumably the subunit molecule contains either an N-terminal or internal amino acid sequence constituting a neutrophil-activating motif (54). This only requires a sequence of amino acids recognized by a receptor on the neutrophil surface. Functional internal peptide sequences involved in receptor binding may consist of as few as three

amino acid residues, such as, for example, the well-known integrin recognition tripeptide Arg-Gly-Asp (RGD) (54) and the ELR sequence which mediates neutrophil receptor binding (30, 45). Interestingly, Owhashi et al. (43) recently cloned and identified a protein chemotactic for neutrophils from the filarial parasite *Dirofilaria immitis*; this protein has an internal chemotactic tripeptide sequence Met-Phe-Lys (MFK) with fMLP-like activity, but like HP-NAP, its effect was not blocked by fMLP antagonists. Bacterial proteins known to activate phagocytes include type 1 pili of *E. coli*, which activate macrophages by a process involving upregulation of CD11b and CD18 (20), fimbriae of *Porphyromonas gingivalis*, a causative agent of chronic inflammatory gum disease, which activates macrophages (26), and the theta toxin of *Clostridium perfringens*, which upregulates leukocyte CD11/CD18 (7). Ongoing studies are aimed at identification of the amino acid motif responsible for interaction of HP-NAP with neutrophils and identification of the target neutrophil receptor.

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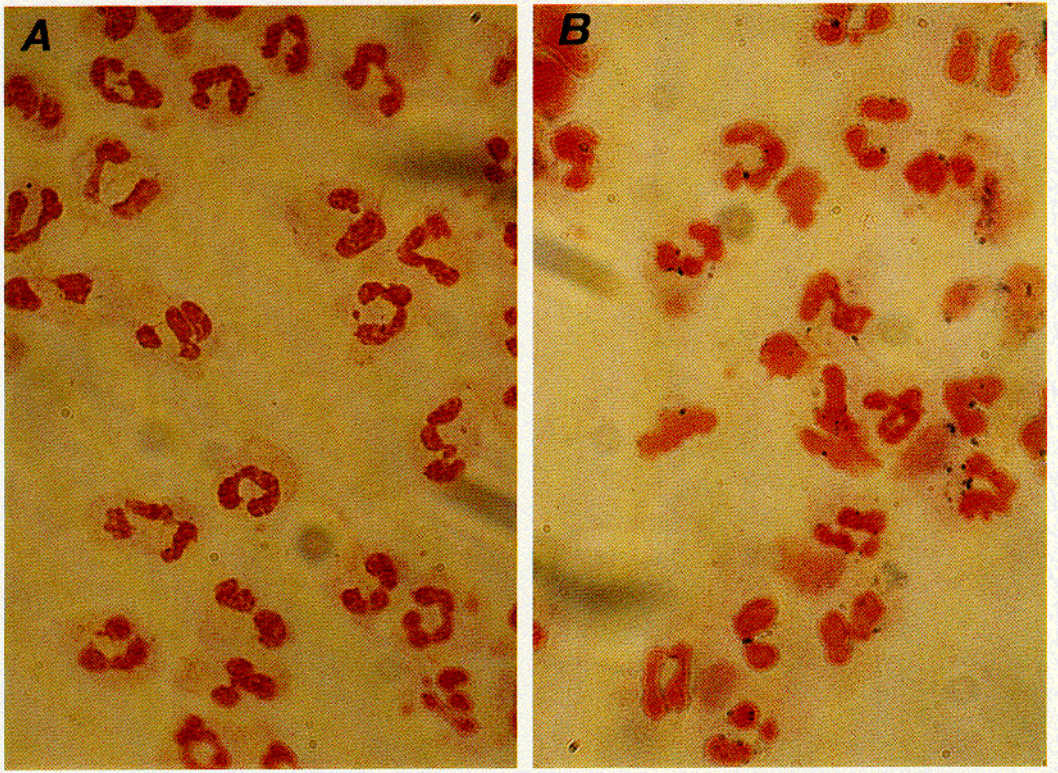
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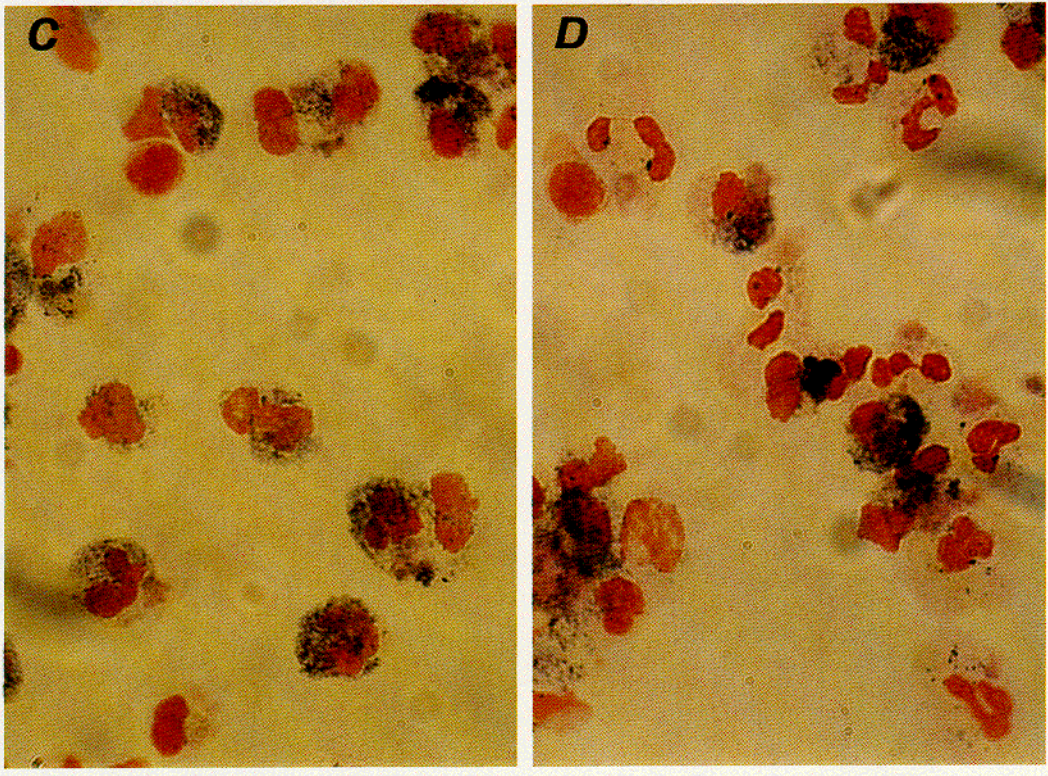
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