Helicobacter pylori dupA Is Polymorphic, and Its Active Form Induces Proinflammatory Cytokine Secretion by Mononuclear Cells

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Background. Infection with Helicobacter pylori possessing a newly described virulence factor—duodenal ulcer–promoting gene A (dupA)—has been associated with duodenal ulceration and increased gastric inflammation.

Methods. The dupA locus of 34 strains was sequenced. A panel of dupA mutants was generated and cocultured with human gastric epithelial cells and peripheral blood mononuclear cells; proinflammatory cytokine release was measured. IL8 expression was measured in human gastric biopsy specimens and related to the dupA and cagA status of infecting strains.

Results. Most H. pylori strains had a dupA allele that was longer (1884 bp; dupA1) than previously described dupA alleles, although some had truncated versions (dupA2). Unlike the best-characterized H. pylori virulence determinant, the cag pathogenicity island (cag PaI), neither dupA type induced release of interleukin (IL)–8 from gastric epithelial cells. However, infections due to dupA–positive strains were associated with higher-level mucosal IL–8 messenger RNA expression in the human stomach than were infections due to dupA–negative strains. To explain this paradox, we found that dupA1 (but not dupA2 or the cag PaI) substantially increased H. pylori–induced IL–12p40 and IL–12p70 production from CD14+ mononuclear cells. Other T helper 1–associated cytokines were also modestly induced.

Conclusion. We suggest that virulent H. pylori strains cause inflammation by stimulating epithelial cells through cag-encoded proteins and mononuclear inflammatory cells through dupA1 products.

Helicobacter pylori is the main cause of peptic ulceration, gastric adenocarcinoma, and gastric lymphoma [1]. Its best-characterized virulence factor is the cag pathogenicity island (cag PaI) [2, 3]. Strains possessing

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dupA Induces CD14+ Cell IL-12 Secretion • JID 2010:202 (15 July) • 261
dupA was PCR amplified using primers DupA-WXF (5′-GATA-TACCATGATGATTTCACTTAACAGAC-3′) and DAR1 (5′-TTAATACTCTCTTATAGTCTTGTGG-3′). A second reverse primer, JHP0919R2 (5′-GCCCATGGGACCCGCAAGAATTAAGAC-3′), was designed within jhp0919 to include the next predicted stop codon, based on the nucleotide sequence from strain J99 and used with DupA-WXF or the previously described primer DupA918F [15]. Amplified genes were sequenced on both strands with the use of BigDye Terminator version 1.1 (Applied Biosystems) on a Perkin-Elmer ABI Prism 3130 fluorescent analyzer.

Mutant construction. We constructed dupA insertion mutants in several different H. pylori strains (Figure 1). Most of dupA was amplified from strain 93-67, with the use of primers DAF1 (5′-GACGATCTATTGAATTATACGCTG-3′) and DAR1, and cloned into pGem-T Easy (Promega). Inverse PCR was used to engineer Xhol and Xmal sites into dupA with the use of primers NHDA2R (5′-CTATATCCCAGGGAATACAGGCT-3′) and NHDA3F (5′-CAATACCTCGAGGCCAATACAGATGTTGTG-3′). The chloramphenicol resistance cassette from pBSC103 [21] was excised (Xhol/Xmal) and ligated into dupA to create plasmid pCDA723. H. pylori strains were transformed with this, and allelic exchange mutants were selected by marker rescue on blood-agar plates containing 30 μg/mL chloramphenicol. Disruption of dupA and confirmation of double crossover were determined by Southern blotting and restriction digestion. Comparative broth cultures of mutants and parental strain pairs demonstrated no differences in growth rates.

We also constructed isogenic cagE insertion mutants in dupA-deficient mutants of strains 93-67 and AB31, as described elsewhere [22].

**Gastric epithelial cell culture.** AGS and ST16 cell lines [23] were grown in nutrient mixture F12 Ham (Invitrogen) containing 10% (vol/vol) fetal bovine serum; MKN45 cells were grown in RPMI 1640 medium with 10% fetal bovine serum and 200 mmol/L l-glutamine (Invitrogen); and MKN28 cells were grown in DMEM/Ham’s F12 mixture with 10% fetal
bovine serum and 200 mmol/L L-glutamine. Cell lines were cultured at 37°C in a humidified atmosphere of 5% carbon dioxide.

**Peripheral blood mononuclear cell isolation.** Twenty-milliliter blood samples obtained from 6 *H. pylori*-negative volunteers were collected into K3 ethylenediaminetetraacetic acid vacutainer tubes (Greiner). Peripheral blood mononuclear cells (PBMCs) were immediately purified using Histopaque 1077 and cultured in RPMI 1640 medium with 10% fetal bovine serum and 200 mmol/L L-glutamine [24].

**Coculture of cells with *H. pylori.** Gastric epithelial cell lines were seeded into 24-well culture plates and grown to 80%-90% confluence. PBMCs were plated at 2 × 10^6 cells/well. *H. pylori* strains, which were grown on blood agar for 24 h, were harvested into 5 mL of cell culture media, and the absorbance was standardized to 0.1 at 600 nm. *H. pylori* was added to cultures at a multiplicity of infection of 100 for epithelial cells and 20 for PBMCs. Coculture assays were performed in 5 replicates per culture condition. Supernatants were removed after 6 h and 48 h of culture, respectively, for cytokine enzyme-linked immunosorbent assays (ELISAs).

**Cytokine ELISAs.** Cytokine concentrations in coculture supernatants prepared from 4 replicate epithelial cell experiments or with PBMCs from 5 donors were measured using human IL-8 (DuoSet; R&D Systems), IL-10, tumor necrosis factor (TNF)-α, interferon (IFN)-γ (eBioscience), and IL-12p40 (Autogen Biosciences) ELISA kits, in accordance with the manufacturers’ instructions. For further investigation of IL-12p40, PBMCs from an additional donor were cocultured with and without *H. pylori*, with the use of 5 replicate wells per culture condition. IL-12p70 and IL-23 ELISA kits (eBioscience) were used. Optical density values were measured at 450 nm and 620 nm, and cytokine concentrations were calculated by reference to a standard curve included on each plate.

**Real-time PCR.** Total RNA was purified using a RNeasy Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized by SuperScript II reverse transcriptase (RT) (Invitrogen) with oligo(dT) primers. RT-PCR was performed on a Rotor-Gene3000 (Corbett Research) with the use of a DyNaMo HS SYBR Green quantitative PCR Kit (New England Biolabs) [24]. No-template controls were included in each cycle. First-stage RT-PCR samples produced without RT from each RNA template were tested in parallel. Analysis used the method of Pfaffl [25]. Relative expression levels of IL8 were determined by normalizing against GAPDH and using an *H. pylori*-negative gastric mucosal cDNA comparator. Positive control human cDNA (BD Biosciences) was included in all assays. Amplification was done over 45 cycles at 95°C for 15 s, at 61°C for 30 s, and at 72°C for 30 s. The primers used were 5′-CTCTTTGG-CAGCCTTTCTGGA-3′ (IL8 forward), 5′-AGTTCTTATAGCCT-CCTGGCA-3′ (IL8 reverse), 5′-CCACATCGCTCAGACAC-CAT-3′ (GAPDH forward), and 5′-GGCAACATATGCCACTTTACCCAGGT-3′ (GAPDH reverse).

**Flow cytometry.** A total of 5 × 10^7 PBMCs were incubated with *H. pylori* at a multiplicity of infection of 20 or in medium alone for 16 h. Cells were stained with anti–CD14-phycocerythrin–cytoxin 5.1 (PC5; AbD Serotec) before staining with anti–IL-12p40–phycocerythrin (PE; eBioscience) or anti–IL-12p70–PE (BD Pharmingen), as described elsewhere [24]. Data on 100,000 events were acquired using a Coulter Epics Alfa flow cytometer. Isotype controls were used to ensure staining specificity. Analysis was performed using Weasel software (version 2.5; Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia).

**Statistics.** Data were analyzed using Minitab 15 software (version 15; Minitab). In box-and-whisker plots, horizontal lines denote median values, boxes denote first to third quartiles, and whiskers denote the lowest and highest observations within 1.5 times the interquartile range. Paired Student’s *t* tests were used to compare the cytokine responses of the 5 PBMC samples in coculture supernatants, and unpaired *t* tests were used when PBMCs obtained from only a single donor were cultured. Unpaired RT-PCR data were compared using Mann-Whitney *U* tests. *P* = .05 was considered to denote statistical significance.

**RESULTS**

**Nucleotide Sequence Analysis of the *H. pylori dupA* Locus**

**Natural polymorphisms within dupA.** To examine *dupA* polymorphisms, we initially sequenced *dupA* from 8 *dupA*-positive *H. pylori* clinical isolates from the United States and the United Kingdom (93-67, 93-68, A101, AB21, AB31, AB43, J123, and J178) (Figures 1 and 2, the latter of which appears only in the electronic version of the *Journal*). Three of these sequences had single-nucleotide deletions that truncated the putative coding region (Figure 1). All 8 had a variation at the 3′ end of *dupA* that was not present in previously described strains C142 or J99 because of an adenine insertion (within a polyadenine stretch), which changed the deduced amino acid sequence between nucleotide 1738 and 1800 (Figure 2). In all but one strain (J123), an additional adenine insertion in a second polyadenine stretch altered the final 10 predicted amino acids and abolished the stop codon described in strains C142 and J99, thus extending the ORFs. To determine the position of the stop codon in these strains, we PCR amplified and sequenced the 3′ region of *dupA* and the 5′ region of the adjacent sequence between nucleotide 1738 and 1800 (Figure 2). In all but one strain (J123), an additional adenine insertion in a second polyadenine stretch altered the final 10 predicted amino acids and abolished the stop codon described in strains C142 and J99, thus extending the ORFs. To determine the position of the stop codon in these strains, we PCR amplified and sequenced the 3′ region of *dupA* and the 5′ region of the adjacent sequence between nucleotide 1738 and 1800 (Figure 2). In all but one strain (J123), an additional adenine insertion in a second polyadenine stretch altered the final 10 predicted amino acids and abolished the stop codon described in strains C142 and J99, thus extending the ORFs. To determine the position of the stop codon in these strains, we PCR amplified and sequenced the 3′ region of *dupA* and the 5′ region of the adjacent sequence between nucleotide 1738 and 1800 (Figure 2). In all but one strain (J123), an additional adenine insertion in a second polyadenine stretch altered the final 10 predicted amino acids and abolished the stop codon described in strains C142 and J99, thus extending the ORFs. To determine the position of the stop codon in these strains, we PCR amplified and sequenced the 3′ region of *dupA* and the 5′ region of the adjacent

**Figure 2.** Polymorphisms in the deduced amino acid sequence at the *dupA* locus from amino acid positions 436–628, for 36 *Helicobacter pylori* strains from various countries.
Figure 3. A, Mean levels of interleukin (IL)–8 secretion from MKN45 cells cocultured with Helicobacter pylori strains (AB31, AB21, 93-67, A101, and J123) and their isogenic dupA mutants for 48 h. The black bar denotes the wild-type strain, and the light gray bar denotes the isogenic dupA mutant. The concentrations of IL-8 in the medium were determined by enzyme-linked immunosorbent assay (ELISA). Error bars denote standard deviations (SD).

B, IL-8 secretion from AGS cells after 48 h of coculture with H. pylori wild-type strains AB31 and 93-67 and their isogenic dupA and cagEp mutants. Black bars denote wild-type strains; light gray bars, dupA mutants; dark gray bars, cagEp mutants; and white bars, double (cagEp and dupA) mutants. The concentrations of IL-8 in the medium were determined by ELISA. Error bars denote standard deviations (SD).

C, IL-8 secretion from AGS cells in response to coculture with wild-type strains of varying dupA and cagEp status. AGS cells were cocultured for 48 h with clinical isolates of H. pylori strains possessing or lacking dupA and possessing or lacking the cagEp gene. *Significantly different from the wild-type strain (P < 0.05).

D, IL8 messenger RNA expression in antral gastric biopsy specimens obtained from patients infected with dupA-positive and dupA-negative H. pylori strains. Real-time polymerase chain reaction was performed, normalizing IL8 expression relative to the housekeeping gene GAPDH and in comparison with complementary DNA prepared from uninfected biopsy specimens, to provide a fold difference. Biopsy specimens obtained from 17 H. pylori-positive patients were tested (6 were dupA positive, and 11 were dupA negative), along with biopsy specimens from 5 uninfected patients as controls. *Significant difference between dupA positive and dupA negative (P < 0.05).

gene jhp0919. In these 7 strains, the stop codon was found at 1884 bp, 5 bp after the putative start codon of jhp0919 (Figures 1 and Figure 2). Thus, of the 8 strains that we initially examined, 4 (strains AB21, AB31, AB43, and 93–68) had a dupA ORF of 1884 bp, longer than previously described dupA ORF; 1 (strain J123) had a dupA ORF of 1839 bp, as described for strain C142 [12] (although dupA differed slightly in its predicted amino acid sequence at the C-terminal end); and 3 had 2 ORFs in the dupA locus (Figures 1 and Figure 2).

The most prevalent form of dupA. Having found considerably more sequence polymorphism than anticipated, we further assessed which forms were most common. Nucleotide sequence analysis of the polymorphic region of dupA (including the 3′ region of jhp0917 and the whole of jhp0918) was performed for 26 other strains from Belgium, South Africa, China, and the United States [15]. Twenty-one strains possessed the new extended form of dupA. Three (J133, GC5, and GC77) possessed 2 separate jhp0917 and jhp0918 ORFs. Strains 32385 and J188 had dupA ORFs of 1500 bp and 1635 bp, respectively (Figure 2). The most common form of dupA among these strains (found in 25 of 34 sequences in total) was the extended form. We have therefore classified dupA into 2 main groups. We hereafter refer to the most common extended ORF within jhp0917–jhp0919 as dupA1, and we refer to dupA with an early stop codon as dupA2. Sometimes this splits the locus into 2 ORFs that are roughly equivalent to jhp0917 and jhp0918 (as found in strain J99), but there are several different variants, raising the possibility that this is a degenerate form.
dupA Induces CD14+ Cell IL-12 Secretion

**Effect of dupA on Secretion of IL-8 from Gastric Epithelial Cells**

dupA and lack of induction of IL-8 secretion from gastric epithelial cells in vitro. Lu et al [12] showed that dupA in strain C142 induced IL-8 production by gastric epithelial cell lines. Therefore, having created isogenic dupA insertion/deletion mutants, we investigated whether dupA1 and dupA2 alleles induced similar levels of IL-8 secretion. We selected 2 dupA1 strains (AB21 and AB31) and 2 dupA2 strains (93-67 and A101) (Figure 1). Strain C142 [12] was not available to us, so we selected a strain with a dupA ORF of the same length (strain J123). All were cag Pal positive. We cocultured these strains or their isogenic dupA mutants with the gastric epithelial cell line MKN45 (as used elsewhere [12]) for 48 h and then quantified IL-8 levels in supernatant (Figure 3A). Similar cultures were performed with 2 dupA1-positive cag Pal–negative wild-type and dupA mutant strains (AB43 and 93-68) (data not shown). Surprisingly, no significant differences were observed between any of the paired wild-types and isogenic mutants. To test this finding more thoroughly, the strains and their dupA mutants were cocultured with gastric epithelial cell lines AGS, ST16, and MKN28. Again, no effects were seen (data not shown).

The profound effect of cag Pal on gastric epithelial cell IL-8 responses to *H. pylori* in the presence and absence of dupA. The cag Pal is the main determinant of *H. pylori*–induced epithelial cell IL-8 release [6, 7, 26]. To see whether cag Pal–induced IL-8 responses were influenced by dupA mutation, we constructed cagE mutants in strains AB31 and 93-67 wild-type and dupA isogenic mutant strains. Coculture with AGS cells for 48 h showed that dupA disruption had no effect on IL-8 production; however, as expected, cagE disruption dramatically reduced it (Figure 3B).

Next, AGS cell IL-8 induction by 20 wild-type *H. pylori* isolates possessing or lacking cagE and/or dupA was assessed. cagE-positive strains induced more IL-8, but dupA status had no effect (Figure 3C).

**Association of dupA with IL8 expression in vivo.** We re-
examined the association between dupA status and IL8 expression in vivo [12]. Quantitative RT-PCR analysis of IL8 messenger RNA was performed on human gastric biopsy specimens obtained from 17 H. pylori-infected patients and 5 H. pylori-negative controls. Colonizing strains were genotyped (6 were dupA positive and 11 were dupA negative) [16]. Median IL8 messenger RNA levels were 5-fold higher for dupA-positive infections than for dupA-negative infections (\( P = .02 \)), and they were very low for uninfected patients (Figure 3D). The difference was still present when only caga-positive samples were considered (6 were dupA positive and 7 were dupA negative; \( P = .03 \)).

**Effect of dupA on Inflammatory Cytokine Responses of Mononuclear Cells**

**dupA and increased H. pylori–induced production of IL-8 and other cytokines by PBMCs.** Because dupA was associated with increased gastric IL8 expression, we hypothesized that dupA interacts with categories of cells other than epithelial cells. Because the bacteria are known to have effects on immune and inflammatory cells [27, 28], we studied the effect of dupA on IL-8 production by PBMCs, and we examined other cytokines known to affect epithelial cell IL-8 production [29, 30].

PBMCs from 5 separate donors were cocultured with dupA-positive strains AB31 and AB21 and their isogenic dupA mutants. After 48 h, IL-8 secretion was slightly but significantly higher in response to wild types than dupA mutants (for AB31, the mean increase was 8% \( P = .023 \)); for AB21, the mean increase was 6% \( P = .024 \)) (Figure 4A). Small but statistically significant differences were observed in IFN-\( \gamma \) (for AB31, the mean increase was 35% \( P = .007 \)); for AB21, the mean increase was 9% \( P = .003 \)) (Figure 4B) and TNFa (for AB31, the mean increase was 4% \( P = .02 \)); for AB21, the mean increase was 8% \( P = .01 \)) (Figure 4C) responses. However, a more marked effect was seen on IL-12p40 secretion, where the wild-type strains induced more than twice the concentrations stimulated by the corresponding dupA mutants (for AB31, the mean increase was 106% \( P = .002 \)); for AB21, the mean increase was 176% \( P = .001 \)) (Figure 4D).

We observed no differences in induction of IL-10 and IL-4 from PBMCs by wild-type H. pylori strains, compared with their matched dupA-negative isogenic mutants (data not shown).

**Effect of dupA polymorphisms on IL-12 and other cytokine responses from PBMCs.** To examine whether dupA2 also in-

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**Figure 5.** Interleukin (IL)–12p40 secretion from peripheral blood mononuclear cells (PBMCs) after 48 h of coculture with *Helicobacter pylori* strains AB31 (dupA1), 93-67 (dupA2), and their isogenic dupA and cagE mutants. Black bars denote wild-type strains; light gray bars, dupA mutants; dark gray bars, cagE mutants; and white bars, double cagE and dupA mutants. The concentrations of IL-12p40 in the medium were determined by enzyme-linked immunosorbent assay (ELISA). Error bars denote standard deviations (\( n = 5 \)). * Significantly different from the wild type (\( P < .05 \)).

**Figure 6.** Interleukin (IL)–12p40 (A), IL-12p70 (B), and IL-23 (C) secretion from peripheral blood mononuclear cells (PBMCs) after 48 h of coculture with *Helicobacter pylori* strains AB31 and AB21 (both dupA1) and their isogenic dupA mutants. Black bars denote wild-type strains, and light gray bars denote dupA mutants. The cytokine concentrations in the medium were determined by enzyme-linked immunoassay. Error bars denote standard deviations (5 parallel cocultures with PBMCs from the same donor). * Significant difference between the wild type and the mutant (\( P < .05 \)).
creases PBMC cytokine production and to compare this with dupA1 effects, we assayed IL-12p40 responses. IL-12p40 release by PBMCs cultured with wild-type dupA1-positive strains was significantly higher than that induced by dupA2 strains ($P < .01$) (Figure 4D). No significant difference in IL-12p40 secretion was observed between dupA2 wild-type and isogenic mutants, implying that dupA2 is inactive (Figure 4D). As expected, dupA2 mutagenesis had no effect on $H. pylori$-induced IFN-γ, TNF-α, or IL-8 secretion from PBMCs (data not shown).

**Effect of dupA and the cag Pal on $H. pylori$–induced IL-12 secretion by mononuclear cells.** PBMCs were incubated with cagE and dupA single and double mutants of strains AB31 and 93-67. As described above, dupA disruption dramatically reduced the IL-12 response; however, in contrast, disruption of cagE had no effect (Figure 5). Thus, dupA, but not an intact cag Pal, enhances $H. pylori$–induced IL-12p40 secretion by mononuclear cells.

**Characterization of dupA1-induced IL-12-related cytokines.** IL-12p40 is a constituent of the heterodimeric proinflammatory cytokines IL-12p70 and IL-23, and it may also exist as a homodimer [31, 32]. We therefore investigated which forms of IL-12p40 were secreted in response to $H. pylori$ (Figure 6). The IL-12p40 response of PBMCs cultured with wild-type strains was again significantly higher than that induced by the corresponding isogenic dupA1 mutants (for AB21, the mean increase was 88% [P = .002]; for AB31, the mean increase was 51% [P = .001]) (Figure 6A). The concentrations, using blood from a different single donor, were higher than those previously observed, but the same trends were present. IL-12p70 levels (Figure 6B) were lower than IL-12p40 levels, but the difference in response to dupA mutant and wild-type strains was more marked (for AB21, the mean increase was 160% [P = .002]; for AB31, the mean increase was 169% [P = .002]). IL-23 responses (Figure 6C) were smaller than IL-12p40 and IL-12p70 responses, but again there was a significant increase in the response to wild-type strains, compared with dupA mutant strains (for AB21, the mean increase was 69% [P = .002]; for AB31, the mean increase was 105% [P = .002]). These results indicate that dupA stimulates increased IL-12p40, IL-12p70, and IL-23 expression from PBMCs.

Monocytes are the most likely source of IL-12p40, IL-12p70, and IL-23 in our PBMC cultures, although a recent report showed that IL-12p40 may be expressed by T cells in response to $H. pylori$ [33]. To confirm the ELISA data, and to determine the cellular source of IL-12, PBMCs were incubated with the AB31 wild-type strain and dupA mutant before immunostaining for CD14, IL-12p40, and IL-12p70 and performing flow cytometry (Table 1). As with the ELISAs, the wild-type induced numerically higher IL-12p40 and IL-12p70 responses than did the dupA-negative mutant. Most IL-12–positive events were CD14+ (73.08% of IL-12p70–positive responses and 50.91% of IL-12p40–positive responses).

**DISCUSSION**

dupA is a recently described $H. pylori$ virulence factor. The exact disease associations are different in different populations [12–15, 34]. Previous nucleotide sequencing of dupA revealed that this gene is naturally polymorphic. We speculated that this might contribute to the population differences and explored dupA polymorphism in some detail.

The most common form of dupA (dupA1) was a previously undescribed extended ORF that encompasses parts of jhp0917, jhp0918, and the very 5′ end of jhp0919 in the genome sequence strain J99. The second most common form (dupA2) contains 2 ORFs, although these vary in their exact start and stop sites. There are also intermediate forms with short or long truncations of dupA1; indeed, the original dupA allele in strain C142 [12] is a truncated form. Currently, no DupA antibodies are available, so we do not know which of these dupA alleles encode proteins. Thus, we used the approach of dupA disruption (in our case, in multiple strains) to explore dupA phenotypes.

Unlike Lu et al [12], we showed that dupA had no effect on $H. pylori$–induced IL-8 production by epithelial cell lines. This

| Table 1. Frequencies of Peripheral Blood Mononuclear Cells (PBMCs) Expressing Interleukin (IL)–12p40 and IL-12p70 and of CD14+ Monocytes after Incubation with the Helicobacter pylori Wild-Type dupA1 Strain AB31 and Its Isogenic dupA Mutant |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Parameter       | IL-12p40        | IL-12p70        |                 |                 |                 |
|                 | Untreated       | $H. pylori$ AB31 wt | $H. pylori$ AB31 ΔdupA | Untreated       | $H. pylori$ AB31 wt | $H. pylori$ AB31 ΔdupA |
| Total % IL-12–positive events | 0.81 | 3.06 | 1.71 | 0.69 | 2.44 | 1.02 |
| Total mean IL-12 FL | 8.3 | 19.3 | 10.3 | 13.2 | 20.6 | 13.1 |
| % CD14+ of IL-12–positive events | 28.4 | 50.91 | 46.42 | 16.04 | 73.08 | 17.07 |
| % IL-12–positive of CD14+ events | 2.61 | 34.59 | 24.60 | 1.31 | 25.67 | 6.28 |

*NOTE.* The percentages of IL-12+ and CD14+ cells are displayed, as is the mean intensity of fluorescence (FL) from IL-12 staining. PBMCs were cultured for 16 h with and without $H. pylori$ before dual staining for CD14 and IL-12 was done. Data on 100,000 events were collected by flow cytometry and were gated for lymphocytes and monocytes from a forward scatter/side scatter plot. Experiments have been performed using PBMCs from 3 individuals; data from one representative experiment are displayed. wt, wild type.
was tested rigorously using 4 different cell lines and 7 panels of isogenic dupA mutants. In support of our findings, nonisogenic data from Schmidt et al [35], who used clinical H. pylori isolates cocultured with AGS cells, showed no association between dupA status and IL-8 release. It remains possible that strain C142, which was used by Lu et al [12], does have an effect not exhibited by other strains. However, strain J123, which was very similar for dupA, did not stimulate increased IL-8 secretion.

After obtaining our negative results on epithelial cells in vitro, we reexamined the association of dupA-positive infection with IL-8 expression in the gastric mucosa. As reported elsewhere [12], we found a strong association, even when correcting for cag PaI status. We therefore hypothesized that the major effect of dupA may be on other cell types, either by directly stimulating IL-8 release or by stimulating the release of other cytokines known to stimulate epithelial cell IL-8 secretion. We concentrated on mononuclear leukocytes as a major source of inflammatory cytokines in the infected gastric mucosa. Using our mutagenesis approach, we found that dupA1 increased H. pylori–induced proinflammatory cytokine production (most markedly, IL-12p40) by these cells, but dupA2 was inactive. Interestingly, the cag PaI, despite its effect on epithelial cell cytokine production and its described association with the development of a Th1 response [36], had no effect on H. pylori–induced mononuclear cell cytokine production.

In addition to its effect on IL-12p40 expression by mononuclear cells, dupA1 also had a marked effect on IL-12p70 and IL-23, both of which contain an IL-12p40 subunit. These proinflammatory factors are associated with H. pylori–induced Th1 [37] and Th17 responses, respectively [38], and with more severe inflammation and disease [39]. The IL-12p40 response was far higher than the IL-12p70 and IL-23 responses; thus, the homodimeric p40/p40 (IL-12p80) form was likely induced. This was originally considered to be an IL-12 antagonist [40] but is now known to act as a chemoattractant for macrophages and bacteria-stimulated dendritic cells and to be associated with inflammatory diseases [32, 41, 42]. The source of IL-12p40 and IL-12p70 was found to be CD14+ cells. Future experiments will investigate the signaling pathways stimulated by dupA1-positive strains in these cells.

Experiments in mice indicate that the quality of the T helper response to H. pylori is critically important for eradication or persistence of the infection and induction or prevention of pathologic findings [43, 44]. Our report suggests that dupA induces gastric inflammation and pathology either directly or indirectly through action upon infiltrating leukocytes rather than upon epithelial cells, and that it may increase the risk of disease by stimulating a more pronounced Th1 response. One outstanding question is whether H. pylori and its virulence factors actually come into contact with inflammatory cells in the gastric mucosa or whether they are always physically separated by the epithelial layer. However, recent studies convincingly report the penetration of H. pylori through the epithelium into underlying tissues [45, 46], perhaps in part after tight junction loosening by CagA and other factors [47].

Our investigations of dupA provide tantalizing new data on H. pylori and its subtle interactions with the human host. Other inflammation-associated virulence factors, such as the cag PaI, may act primarily through inducing proinflammatory cytokine secretion by gastric epithelial cells, leading to inflammatory cell infiltration into the gastric mucosa. dupA appears to act at the next level: it stimulates those inflammatory cells to influence the type of immune response, possibly skewing this toward Th1. This finding further demonstrates the complexity of the interactions of H. pylori with humans, which now appear to be multilayered.

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268 • JID 2010:202 (15 July) • Hussein et al


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Note added in proof. Since the submission of our manuscript, new findings indicate that the dupA allele of some strains might have an extended 5′ end. To support our results, we analyzed the 5′ end of dupA by polymerase chain reaction and sequencing from strains AB21 and AB31 using primers DASseqfwd (5′-ATGGCGTGGTGCTATTGGTG-3′) and DASseqrev (5′-TTCGCATCGGTCATCGTGC-3′). These strains have a positive dupA phenotype in coculture experiments. We successfully confirmed that the dupA allele in these strains is indeed extended at the 5′ end, leading to a total length of 2499 bp.