

# Induction of Gastric Epithelial Cell Apoptosis by *Helicobacter pylori* Vacuolating Cytotoxin<sup>1</sup>

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## ABSTRACT

Chronic gastritis induced by *Helicobacter pylori* is a strong risk factor for the development of distal gastric adenocarcinoma. A specific host response to *H. pylori* that may contribute to gastric carcinogenesis is epithelial cell apoptosis. The aim of this study was to investigate the capacity of *H. pylori* vacuolating toxin (VacA) to induce gastric epithelial cell apoptosis. When cocultured with AGS gastric epithelial cells, *H. pylori* strain 60190, which expresses a type s1/m1 VacA toxin, induced significantly higher levels of apoptosis than did an isogenic *vacA* null mutant strain. VacA purified from strain 60190 induced apoptosis in a dose-dependent manner, which required acid activation of the purified toxin and the presence of ammonium chloride. In contrast, apoptosis was not induced after incubation with a chimeric s2/m1 toxin (in which the s1 sequence at the NH<sub>2</sub> terminus of VacA from strain 60190 was replaced with the s2 sequence from the nontoxic strain Tx30a) or a VacA mutant protein (VacA Δ6–27) that lacks a unique strongly hydrophobic region near the VacA NH<sub>2</sub> terminus. Moreover, when an equimolar mixture of purified VacA Δ6–27 and purified wild-type VacA were added simultaneously to AGS cells, the mutant toxin exhibited a dominant negative effect, completely inhibiting the apoptosis-inducing activity of wild-type VacA. These results indicate that VacA induces gastric epithelial cell apoptosis and suggest that differences in levels of gastric mucosal epithelial apoptosis among *H. pylori*-infected persons may result from strain-dependent variations in VacA structure.

## INTRODUCTION

Gastric adenocarcinoma is the second leading cause of cancer-related death in the world (1). Epidemiological studies in humans, as well as experimental infections in rodents, have demonstrated that chronic gastritis induced by *Helicobacter pylori* is a strong risk factor for this malignancy (2–8); however, only a fraction of individuals who carry *H. pylori* ever develop gastric neoplasia. Variations in cancer risk among *H. pylori*-infected persons may be related to differences among *H. pylori* strains, variable host characteristics (e.g., IL-1β<sup>3</sup> polymorphisms), environmental influences, and/or specific interactions between host and microbial determinants.

One strain-specific *H. pylori* locus that has been associated with an increased risk for carcinogenesis is *vacA*, which encodes a secreted bacterial toxin (VacA; Refs. 9–14). When added to mammalian cells *in vitro*, VacA induces multiple structural and functional alterations in cells, the most prominent of which is the formation of large intracellular vacuoles (15). Vacuole formation in response to purified VacA is dependent on the presence of weak bases such as ammonia, as well as on internalization of the toxin by cells (16–21).

A *vacA* gene is present in virtually all of the *H. pylori* strains examined (10, 22); however, strains vary considerably in the production of vacuolating cytotoxin activity. This variation is primarily attributable to variations in *vacA* gene structure. The regions of greatest diversity are localized near the 5' end of *vacA* (allele families s1a, s1b, s1c, or s2) and in the mid-region of *vacA* (allele families m1 or m2; 22–24). Most type s1 VacA toxins possess detectable vacuolating cytotoxic activity *in vitro*, whereas type s2 VacA proteins possess little if any cytotoxic activity (22). This is attributable to the presence of a 12-amino-acid hydrophilic segment at the NH<sub>2</sub> terminus of type s2 toxins, which abolishes cytotoxic activity (25, 26).

*H. pylori* strains that possess a type s1/m1 *vacA* allele are associated with an increased risk of gastric cancer (27–30) and enhanced gastric epithelial cell injury (31, 32) compared with *vacA* s2/m2 strains. This relationship between s1/m1 alleles and gastric cancer is consistent with investigations that have examined the distribution of *vacA* genotypes throughout the world. In regions in which the background rate of distal gastric cancer is high, such as Colombia and Japan, most *H. pylori* strains contain type s1/m1 alleles (24).

Host responses to the presence of *H. pylori* are likely to be important in affecting the threshold for carcinogenesis. In several studies, *H. pylori* has been associated with increased levels of apoptosis in human gastric mucosa (33–36). The capacity of *H. pylori* to induce apoptosis of gastric epithelial cells also has been demonstrated in murine (37–39) and Mongolian gerbil (40, 41) models of infection.

Cell culture model systems have been used to investigate mechanisms through which *H. pylori* induces apoptosis. These studies have demonstrated that multiple *H. pylori* factors, including urease (42), products of the *cag* pathogenicity island (43), and lipopolysaccharide (44) contribute to apoptosis. *H. pylori* urease has been shown to bind to class II MHC molecules and induce apoptosis in KatoIII and N87 gastric epithelial cells (42). *H. pylori* can also stimulate apoptosis *in vitro* by inducing expression of the cell-surface receptor Fas and Fas ligand (36, 45–47), or by activating the transcription factor NF-κB (48).

In addition to these pathways, several lines of evidence suggest that *H. pylori* VacA may induce gastric epithelial cell apoptosis. In a previous study, we demonstrated that a wild-type VacA-producing strain induced apoptosis of gastric epithelial cells to a greater extent than did an isogenic mutant strain, based on flow cytometry analysis (43). Another study has shown that broth culture supernatant from a toxin-producing *H. pylori* strain induced apoptosis of AGS cells (49). However, at present, it is unclear whether VacA alone is sufficient to induce apoptosis or whether additional *H. pylori* factors are required. Thus, the goal of this study was to investigate further the capacity of VacA to induce gastric epithelial cell apoptosis.

## MATERIALS AND METHODS

### *H. pylori* Wild-Type and Isogenic Mutant Strains

*H. pylori* strain 60190 (ATCC 49503) was the parental wild-type strain used in this study; characteristics of its *vacA* gene and corresponding secreted VacA protein have been reported previously (9, 10, 50, 51). *H. pylori* strain 60190-v1 is an isogenic mutant strain in which *vacA* was disrupted by insertion of a

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<sup>3</sup> The abbreviations used are: IL, interleukin; NF, nuclear factor; FBS, fetal bovine serum.

kanamycin cassette (10, 43). This null mutant strain does not express or secrete any detectable VacA protein (10, 43). *H. pylori* strain VM083 is an isogenic mutant strain that secretes a chimeric s2/m1 VacA protein (26) and *H. pylori* strain AV452 is an isogenic mutant strain that secretes a VacA protein (VacA  $\Delta$ 6–27) containing a 22-amino-acid internal deletion near the NH<sub>2</sub> terminus (52). An isogenic *ureB* mutant was generated by insertional mutagenesis as previously described (43), using pMK180, which contains *aphA* (conferring kanamycin resistance) cloned into *ureB* (53). Isogenic *ureB* mutants were selected on Brucella agar with kanamycin (25  $\mu$ g/ml) and confirmed to lack urease activity.

### H. pylori Culture

For coculture experiments with gastric epithelial cells, *H. pylori* were grown in Brucella broth with 5% FBS for 24–48 h, harvested by centrifugation (2000  $\times$  g), and resuspended in antibiotic-free RPMI 1640 with 10% FBS to a concentration of  $1 \times 10^8$  colony forming units/ml. For all of the experiments, *H. pylori* were added to cells at a bacteria:cell concentration of 100:1, based on previous reports that *H. pylori* reproducibly induce apoptosis in AGS cells at this ratio (43, 45). Bacteria were identified as *H. pylori* by urease and oxidase activity as well as by Gram's stain morphology.

### Preparation of H. pylori Broth Culture Supernatants and VacA Purification

*H. pylori* strains were grown in sulfite-free broth (50) containing either 5% FBS or 0.5% activated charcoal. Cultures were incubated on a rotary shaker for 48 h at 37°C in ambient air containing 5% CO<sub>2</sub>, and the cultures then were centrifuged. Broth culture supernatants from cultures containing FBS were concentrated 30-fold by ultrafiltration (Millipore) and passed through a 0.2- $\mu$ m filter. VacA was purified from broth culture supernatants as described previously (50) and then was dialyzed in PBS. Protein concentrations were determined using a Micro-BCA assay (Pierce). Acid activation of VacA was accomplished by dropwise addition of 250 mM HCl to the purified toxin until a pH of 3.0 was reached (54).

### Cell Culture

AGS human gastric epithelial cells (ATCC CRL 1739) were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FBS and 20  $\mu$ g/ml gentamicin in an atmosphere of 5% CO<sub>2</sub> at 37°C. Coculture experiments, in which AGS cells were incubated with viable *H. pylori*, were performed in antibiotic-free media containing 10% FBS, using 12- or 96-well polypropylene tissue culture plates (Nunc, Denmark). AGS cells were not serum starved, and remained subconfluent during each assay. Purified, acid-activated VacA preparations were added to AGS cells in medium with or without 5 mM ammonium chloride. The addition of ammonium chloride did not change the pH of the tissue culture medium.

### Assessment of Apoptosis

**DNA Fragmentation ELISA.** DNA fragmentation was quantified using a commercially available ELISA (Roche, Indianapolis, IN) that detects nucleosomal fragments in cytoplasmic fractions of cells undergoing apoptosis but not in cells undergoing necrosis (41). For these experiments,  $5 \times 10^3$  AGS cells/well in 96-well plates were incubated in triplicate with *H. pylori* ( $5 \times 10^5$  colony forming units/well), filtered supernatants, purified VacA, or medium alone for 24–48 h. Cells were then lysed according to the manufacturer's instructions and centrifuged, and supernatants were analyzed by ELISA exactly as described previously (41). Absorbance was measured at 405 nm.

**DNA-specific Fluorochrome Staining.** AGS cells were cultured with or without purified VacA for 48 h, fixed with ice-cold methanol for 10 min, incubated with 1  $\mu$ g/ml propidium iodide for 5 min, and visualized by fluorescence microscopy. For each sample, at least 100 cells were evaluated by an independent observer (U.S.K.) unaware of experimental conditions. Results are expressed as the mean number of cells with highly condensed nuclei and fragmented chromatin/total number of cells counted.

### Statistics

Results are expressed as mean  $\pm$  SD. The Mann-Whitney *t* test was used for statistical analyses of intergroup comparisons. *P* values of  $\leq 0.05$  were considered significant.

## RESULTS

### VacA Expression by H. pylori Induces Apoptosis in AGS Cells.

As a first step in investigating the capacity of VacA to induce apoptosis, we cocultured AGS cells with the wild-type toxigenic strain 60190 or an isogenic *vacA* null mutant strain. Because VacA-dependent cell vacuolation is potentiated by the presence of weak bases such as ammonia (16–21), we performed these experiments both in the presence and in the absence of ammonium chloride (5 mM). After incubation of AGS cells with live *H. pylori* strains for 24 or 48 h, we measured the extent of DNA fragmentation present in AGS cytoplasmic fractions. The incubation of cells with parental strain 60190 resulted in a significant increase in the release of oligonucleosomal fragments into the cytoplasm at both 24 and 48 h (*P* < 0.05 for each time point; Fig. 1), compared with AGS cells incubated in medium alone. In contrast, coculture with the *vacA* null mutant did not significantly increase apoptosis compared with controls (Fig. 1). The addition of exogenous ammonium chloride did not alter apoptosis induced by the live wild-type *H. pylori* strain (Fig. 1). Because *H. pylori* urease has been reported to induce apoptosis in other experimental systems (42), we also tested an isogenic urease-negative mutant of 60190 and found that apoptosis after coculture with the urease-negative mutant did not differ substantially from apoptosis after coculture with the wild-type strain (data not shown).

We next analyzed the capacity of filtered broth culture supernatants derived from the wild-type and *vacA* null mutant strain to induce apoptosis in AGS cells. *H. pylori* culture supernatants were added to AGS cells and were incubated for 48 h in either the presence or the absence of supplemental ammonium chloride (5 mM), and apoptosis was analyzed by DNA fragmentation ELISA. In the presence or absence of ammonium chloride, only supernatant harvested from the wild-type strain 60190 significantly increased release of nucleosomal fragments (Fig. 2). These findings indicate that culture supernatant from a toxigenic *H. pylori* strain, but not from its isogenic *vacA* null

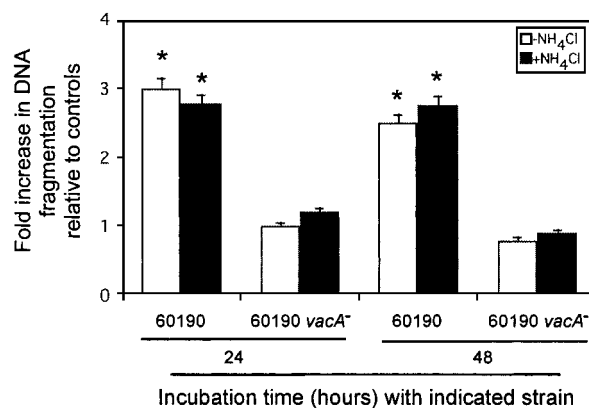


Fig. 1. Apoptosis in AGS cells after incubation with viable *H. pylori* wild-type strain 60190 or an isogenic *vacA* null mutant strain in the presence or absence of ammonium chloride as assessed by DNA fragmentation analysis. AGS cells ( $5 \times 10^3$ /well) were incubated with *H. pylori* strain 60190 or its *vacA* null mutant strain ( $5 \times 10^5$  bacteria/well) for 24–48 h in the presence (solid columns) or absence (open columns) of supplemental ammonium chloride (5 mM). DNA fragmentation was quantified by ELISA. Results represent at least two independent experiments performed in triplicate and are expressed as mean levels of nucleosomal release relative to control cells that were incubated in tissue culture medium without *H. pylori*. Bars, SD; \*, *P* < 0.05 versus AGS cells in medium without *H. pylori*.

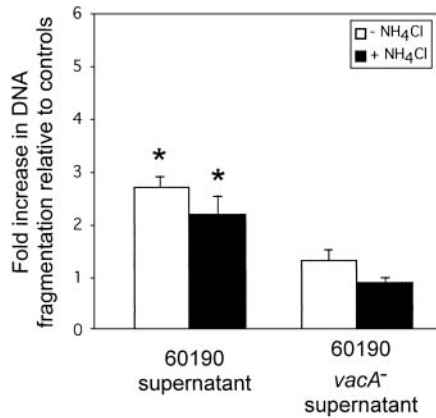


Fig. 2. Filtered supernatants from *H. pylori* wild-type strain 60190, but not an isogenic *vacA* null mutant strain, induce apoptosis in AGS cells. AGS cells were grown alone or in the presence of filtered supernatants from broth cultures of wild-type 60190 or the *vacA* null mutant. Tissue culture medium was either supplemented with 5 mM ammonium chloride (*solid columns*) or not supplemented (*open columns*) as described in "Materials and Methods." After 48 h of incubation, DNA fragmentation was quantified by ELISA. Results represent two independent experiments performed in triplicate and are expressed as the levels of nucleosomal release relative to controls. Data represent mean  $\pm$  SD. \*,  $P < 0.05$  versus AGS cells in medium without additives.

derivative, can induce DNA fragmentation, a phenotype consistent with apoptosis.

**Induction of Apoptosis by Purified VacA.** Purified VacA derived from *H. pylori* broth culture supernatants induces vacuolation of mammalian cells, but this phenotype requires acid activation or alkaline activation of the purified toxin before contact with cells, as well as the presence of supplemental ammonium chloride in the tissue culture medium that overlies the cells (21, 50). To determine whether VacA-dependent apoptosis was dependent on the same conditions required for vacuolating cytotoxic activity, we incubated AGS cells with acid-activated or untreated purified VacA in the presence or absence of supplemental ammonium chloride and examined apoptosis by DNA fragmentation ELISA. In preliminary experiments, the effects of purified VacA on apoptosis in this system were most prominent at 48 h (data not shown); therefore, we focused our subsequent analyses on events occurring at this time point. In the presence of ammonium chloride, acid-activated VacA, but not untreated VacA, significantly increased DNA fragmentation compared with buffer-treated cells or cells incubated with medium alone (Fig. 3). In contrast, in the absence of ammonium chloride, VacA failed to increase DNA

fragmentation (Fig. 3). As expected, treatment of purified VacA with protease (attached to agarose beads) abolished the capacity of the preparation to induce apoptosis, which indicates that the apoptotic activity was attributable to a protein and not attributable to any potential nonprotein contaminants, such as lipopolysaccharide.

To further assess apoptosis, we incubated AGS cells with acidified VacA in the presence of supplemental ammonium chloride and examined nuclear morphology in propidium iodide-stained cells. Incubation of cells with acid-activated VacA, but not acidified buffer, induced condensation of chromatin as well as nuclear segmentation (Fig. 4A), features consistent with apoptosis. Under the experimental conditions of this study, VacA induced detectable nuclear morphological abnormalities in  $28 \pm 6\%$  of cells (Fig. 4B). Thus, results of both a DNA fragmentation assay and propidium iodide staining indicate that purified VacA can induce apoptosis in AGS cells, but these events require acid activation of the purified toxin and the presence of supplemental ammonium chloride.

To determine whether the effects of purified VacA on apoptosis were dose dependent, we next incubated AGS cells with various concentrations of purified acid-activated VacA, in the presence of 5 mM ammonium chloride. Compared with acidified buffer controls, acidified VacA significantly increased apoptosis in a dose-dependent manner, and the threshold concentration required for the induction of programmed cell death was  $>0.1 \mu\text{g/ml}$  (Fig. 5).

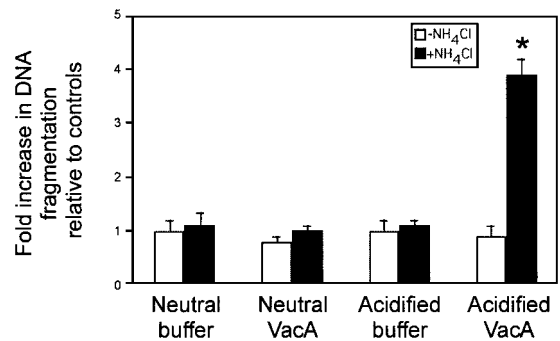


Fig. 3. Apoptosis in AGS cells after incubation with purified VacA. AGS cells were grown alone, in the presence of purified VacA (either acid-activated or untreated,  $5 \mu\text{g/ml}$ ), or in the presence of buffer controls. Tissue culture medium was either supplemented with 5 mM ammonium chloride (*solid columns*) or not supplemented (*open columns*) as described in Methods. After 48 h of incubation, DNA fragmentation was quantified by ELISA. Results are expressed as the levels of nucleosomal release relative to controls. Data represent mean  $\pm$  SD of at least 3 independent experiments. \*,  $P < 0.05$  versus AGS cells in medium without additives.

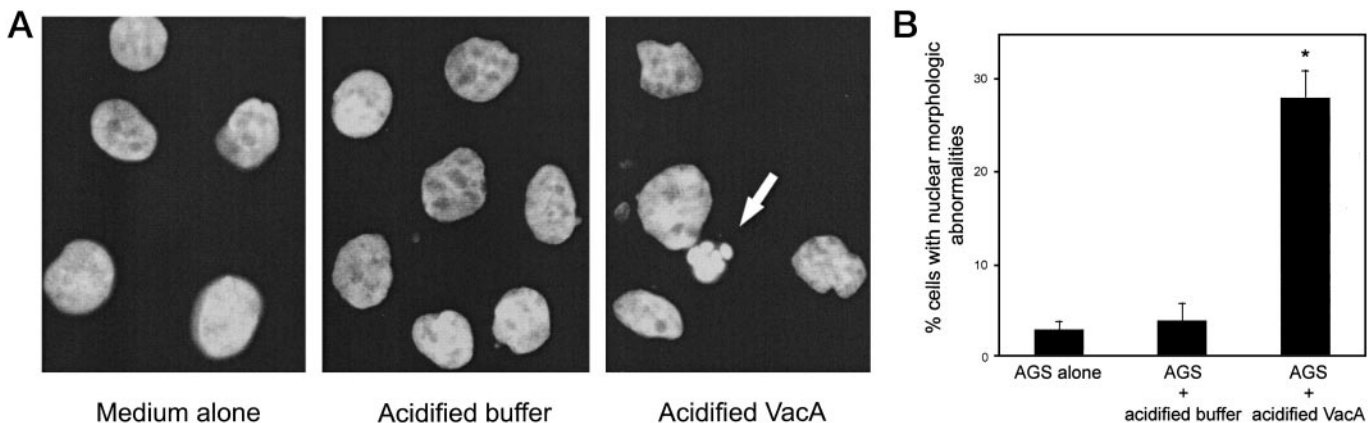


Fig. 4. Incubation with acid-activated VacA induces morphological features consistent with apoptosis as assessed by propidium iodide staining. A, AGS cells ( $1.5 \times 10^5$  cells/well) were grown in 12-well plates in tissue culture medium containing 5 mM ammonium chloride alone, in the presence of acidified buffer, or in the presence of acidified VacA ( $5 \mu\text{g/ml}$ ) for 48 h. Cells were then fixed with methanol, stained with the DNA-specific fluorochrome propidium iodide, and visualized using fluorescent microscopy. Arrow, a representative apoptotic cell. B, scores reflect the mean proportion of cells with nuclear abnormalities as defined by condensed chromatin and nuclear segmentation from two independent experiments. \*,  $P < 0.05$  versus AGS cells in medium without additives. Data represent mean  $\pm$  SD.

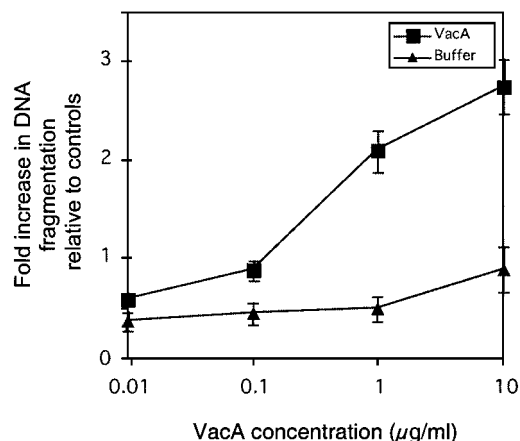


Fig. 5. Dose-dependent effect of VacA on AGS cell apoptosis. AGS cells were incubated for 48 h with various concentrations (0.01–10 µg/ml) of acidified VacA (■) or equal volumes of acidified buffer (▲) in the presence of ammonium chloride (5 mM). Apoptosis was quantified by DNA fragmentation ELISA and results are expressed as the levels of nucleosomal release relative to AGS cells in medium without additives. Data represent results from two independent experiments performed in triplicate and are expressed as mean ± SD.

**Analysis of Type s2 VacA Toxin.** Purified type s1 VacA toxins induce vacuolation of multiple epithelial cell lines *in vitro*, whereas type s2 VacA proteins lack detectable vacuolating activity (22, 26, 55). To test experimentally whether the presence of a type s2 VacA sequence similarly diminishes the capacity of VacA to induce apoptosis, we incubated AGS cells with buffer alone, acidified wild-type s1/m1 VacA from strain 60190 (5 µg/ml), or a chimeric s2/m1 toxin (5 µg/ml) in which the s1 sequence of VacA from strain 60190 was replaced with the s2 sequence from the nontoxicogenic *H. pylori* strain Tx30a (26). Cells were incubated with these VacA preparations for 48 h in the presence of ammonium chloride, and apoptosis was quantified by ELISA. As expected, incubation of cells with acid-activated wild-type VacA induced marked cellular vacuolation and significantly increased apoptosis compared with buffer-treated controls (Fig. 6). In contrast, the s2/m1 VacA protein induced relatively little cell vacuolation, and apoptosis was not altered by the acidified s2/m1 chimera (Fig. 6).

**A Mutant VacA Toxin That Lacks Vacuolating Activity Fails to Induce Apoptosis.** We have previously described the characteristics of a mutant VacA protein (VacA Δ6–27) that lacks a critical hydrophobic region near the VacA NH<sub>2</sub> terminus (52). VacA Δ6–27 is secreted by *H. pylori* and does not exhibit any defects in binding or uptake by cells; however, it is unable to induce vacuolation (52). Because addition of a type s2 sequence to the VacA NH<sub>2</sub> terminus abolished the ability to stimulate apoptosis (Fig. 6), we next investigated whether VacA Δ6–27 could induce apoptosis. AGS cells were incubated with acidified wild-type VacA, VacA Δ6–27, or buffer alone for 48 h in the presence of ammonium chloride; and apoptosis was quantified by ELISA. As expected, based on previous experiments with HeLa cells, wild-type VacA induced extensive vacuolation of AGS cells, whereas VacA Δ6–27 did not (data not shown), confirming that this hydrophobic region was required for cell-vacuolating activity (52). VacA Δ6–27 also lacked the capacity to induce apoptosis (Fig. 6).

**VacA Δ6–27 Exerts a Dominant Negative Effect on Apoptosis Induced by Wild-Type VacA.** We have shown previously that when an equimolar mixture of wild-type VacA and VacA Δ6–27 are added to HeLa cells, the mutant toxin exhibits a dominant negative effect, completely inhibiting the vacuolating activity of wild-type VacA (52). Having established that VacA Δ6–27 failed to stimulate apoptosis, we

next tested the possibility that VacA Δ6–27 could inhibit apoptosis induced by wild-type VacA. As expected, acid-activated wild-type toxin significantly increased apoptosis compared with buffer-treated controls (Table 1). When premixed with wild-type VacA at ratios ranging from 1:1 to 1:16, acid-activated VacA Δ6–27 inhibited DNA fragmentation induced by wild-type VacA in a dose-dependent manner; complete inhibition of apoptotic activity was detected when the ratio of mutant:wild-type VacA was 1:2 (data not shown). These cocultivation experiments indicate that VacA Δ6–27 can effectively block the apoptotic activity of wild-type VacA and suggest that the molecular mechanisms underpinning VacA-induced vacuolation also regulate VacA-dependent apoptosis.

## DISCUSSION

Gastric adenocarcinoma is strongly associated with the presence of *H. pylori* (1–8). One mechanism by which *H. pylori* may augment the risk for carcinogenesis is by altering cellular turnover. Mucosal hyperproliferation has been demonstrated within *H. pylori*-infected gastric tissue (56–58), and multiple studies have concluded that *H. pylori* is also associated with increased levels of apoptosis *in vivo* (33–36). However, another study has concluded that *H. pylori* infection is not associated with increased apoptosis (59). Explanations for this discordance may include various environmental influences and diversity among infecting *H. pylori* strains. To investigate the relationship between *H. pylori* and apoptosis, *in vitro* assays are very useful because multiple variables can be carefully controlled. By using an *in vitro* system to study interactions of *H. pylori* with mammalian cells,

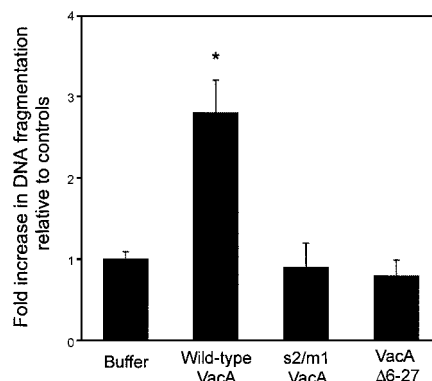


Fig. 6. Apoptosis in AGS cells after incubation with purified wild-type s1/m1 VacA, a chimeric s2/m1 VacA protein, or VacA Δ6–27 as assessed by DNA fragmentation analysis. AGS cells were grown alone or in the presence of acidified buffer, wild-type VacA (5 µg/ml), s2/m1 VacA (5 µg/ml), or VacA Δ6–27 (5 µg/ml). Tissue culture medium was supplemented with ammonium chloride (5 mM) as described in "Materials and Methods." After 48 h of incubation, DNA fragmentation was quantified by ELISA. Results are expressed as the levels of nucleosomal release relative to controls. Data represent mean ± SD of at least two independent experiments. \*,  $P < 0.05$  versus AGS cells in medium without additives.

Table 1 Inhibition of VacA-dependent apoptosis by VacA Δ6–27<sup>a</sup>

Sample <sup>b</sup>	Fold increase in DNA fragmentation relative to:	
	AGS alone <sup>c</sup>	AGS + buffer <sup>c</sup>
Wild-type VacA (5 µg/ml)	6.5 ± 0.4 <sup>d</sup>	6.5 ± 0.2 <sup>d</sup>
VacA Δ6–27 (5 µg/ml)	0.8 ± 0.1	0.6 ± 0.06
Wild-type VacA (5 µg/ml) + VacA Δ6–27 (5 µg/ml)	0.9 ± 0.01	1.4 ± 0.3

<sup>a</sup> Apoptosis was determined by DNA fragmentation ELISA after 48 h of coculture in the presence of ammonium chloride (5 mM).

<sup>b</sup> Wild-type VacA and VacA Δ6–27 were acid-activated before incubation with AGS cells.

<sup>c</sup> Mean ± SD of two independent experiments.

<sup>d</sup>  $P < 0.05$  versus controls.

we have shown that VacA induces apoptosis in AGS gastric epithelial cells. Contact between *H. pylori* and AGS cells also results in activation of NF- $\kappa$ B and induction of IL-8 (60–62), similar to the effects of *H. pylori* observed within colonized gastric mucosa (60, 63). Therefore, this *in vitro* system provides useful insights into the interaction of *H. pylori* with gastric epithelial cells in the human stomach.

We and others have previously reported that VacA contributes to the induction of apoptosis in AGS cells by demonstrating a loss of phenotype using isogenic *vacA* null mutant strains (43, 49). In the present study, we demonstrate that purified VacA can induce apoptosis in the absence of other *H. pylori* factors. Importantly, acid-activation of VacA was required in order for the purified toxin to exert its apoptotic effects. In previous studies, we have shown that acidification of VacA results in disassembly of the toxin's oligomeric structure and enhances its internalization into mammalian cells (21, 50). Thus, it seems likely that the internalization of VacA may be required for the toxin to induce apoptosis. Galmiche *et al.* (64) provided additional insights into these events by showing that VacA fragments that are expressed in transiently transfected nongastric (HeLa) cells, insert into mitochondrial membranes, induce cytochrome *c* release, and activate the caspase-3-dependent cell-death-signaling cascade. These findings are consistent with recent observations that activated caspase-3 is increased within gastric mucosa of *H. pylori*-infected humans and monkeys compared with uninfected tissue (65). Interestingly, *Staphylococcus aureus*  $\alpha$ -toxin, a soluble pore-forming toxin, has been reported to induce mitochondrial release of cytochrome *c* into the cytoplasm of lymphocytes, which is followed by the sequential activation of caspases 8, 9, and 3, ultimately leading to apoptosis (66). Although there may be similarities between the mechanisms by which VacA and *S. aureus*  $\alpha$ -toxin induce apoptosis, the specific intracellular signaling cascades that are activated by full-length VacA in gastric epithelial cells remain to be determined.

In the present studies, supplementation of tissue culture medium with ammonium chloride was required in order for purified VacA to induce apoptosis. Similarly, supplemental ammonium chloride is required for purified VacA to induce cell vacuolation. Recent studies have reported that ammonium alone is able to induce apoptosis in gastric epithelial cells (67, 68); and in one study, ammonium-induced apoptosis was accompanied by cytochrome *c* release and the activation of caspases 9 and 3 (68). *H. pylori* produces ammonia via multiple pathways, one of the most important of which is urease-mediated hydrolysis of urea (69). In addition to generating ammonia, urease has been shown to induce apoptosis in KATOIII and N87 gastric epithelial cells *in vitro* by binding to MHC class II antigens (42), which are expressed on gastric epithelial cell surfaces and up-regulated in the presence of *H. pylori* (70). However, inactivation of *ureB* in the present study had no effect on the ability of *H. pylori* to induce apoptosis in AGS cells. Therefore, the capacity of urease to induce apoptosis, either directly by binding to MHC class II antigens or indirectly by generating a requisite cofactor (ammonia) for VacA-dependent apoptosis, may be cell-line dependent.

Two forms of VacA that fail to induce cell vacuolation (VacA  $\Delta$ 6–27 and s2/m1 VacA; Refs. 26 and 52) also failed to induce apoptosis. In comparison with wild-type VacA, these two inactive forms each contain a mutation or modification near the NH<sub>2</sub> terminus of the toxin (26, 52). This suggests that functions attributable to the NH<sub>2</sub> terminus of VacA are required for both cell-vacuolating and apoptosis-inducing activity. VacA  $\Delta$ 6–27 and s2/m1 VacA each form anion-selective membrane channels significantly less efficiently than does wild-type VacA (26, 52). Thus, it seems likely that membrane channel formation may be required for both VacA-induced cell vacuolation and VacA-induced apoptosis. In previous studies, we have shown that VacA  $\Delta$ 6–27 acts as a dominant negative mutant protein

(26, 52). We now show that, in addition to inhibiting the vacuolating activity of wild-type VacA, this dominant negative mutant inhibits the apoptotic activity of wild-type VacA. The mechanism of inhibition likely involves the formation of hetero-oligomeric complexes, comprised of both wild-type VacA and mutant VacA (26), which are defective in both vacuolating activity and apoptotic activity.

Although VacA and various other *H. pylori* factors can independently induce apoptosis in isolated *in vitro* systems, apoptosis *in vivo* is likely to be influenced by numerous host mediators present within inflamed mucosa. IFN- $\gamma$ , a cytokine that is increased within colonized mucosa, is synergistic with *H. pylori* in inducing Fas-Fas ligand-regulated apoptosis of gastric epithelial cells *in vitro* (45, 71). *H. pylori* infection of IFN- $\gamma$ -deficient mice leads to decreased levels of gastric inflammation compared with levels in infected wild-type mice (72), and *Helicobacter felis* infection of Fas-deficient mice is also associated with reduced levels of inflammation compared with levels in infected wild-type mice; mucosal apoptosis scores are decreased in parallel (38). In contrast, *H. felis* infection of mice lacking secretory phospholipase A<sub>2</sub> leads to increased levels of epithelial cell apoptosis compared with levels in infected wild-type mice (37). We have recently shown that *H. pylori* can induce apoptosis in gastric epithelial cells *in vitro* by activating the transcription factor NF- $\kappa$ B (48), and activated NF- $\kappa$ B is present within gastric epithelial cells of infected but not of uninfected persons (60). IL-1 $\beta$ , which is increased within inflamed mucosa (73), can stimulate multiple intracellular signaling pathways involved in apoptosis, including Fas and NF- $\kappa$ B (45, 60, 71). Thus, differing levels of IL-1 $\beta$  expression within *H. pylori*-colonized gastric mucosa, associated with host-specific IL-1 $\beta$  polymorphisms (74), may contribute to various levels of apoptosis *in vivo*.

The development of intestinal-type gastric adenocarcinoma involves progression through a well-defined series of histological steps, initiated by the transition from normal mucosa to chronic superficial gastritis, followed by the appearance of atrophic gastritis and intestinal metaplasia, and, finally, dysplasia and adenocarcinoma (1). The risk for developing gastric cancer is >90-fold higher in patients with severe multifocal atrophic gastritis than in patients with normal mucosa (75). Apoptosis in response to VacA and other *H. pylori* factors may play an important role in the process by which gastric cancer develops in *H. pylori*-infected humans. The exact mechanisms by which *H. pylori*-associated apoptosis may predispose to gastric cancer are not yet entirely clear, but enhanced rates of cell loss could potentially accelerate the development of gastric atrophy or intestinal metaplasia (76). In addition, apoptosis may contribute to compensatory hyperproliferation, which may also increase the risk for gastric cancer.

In conclusion, the present studies provide strong evidence that VacA stimulates apoptosis in AGS cells. Addition of a type s2 sequence to the VacA NH<sub>2</sub> terminus abolishes the capacity of the toxin to stimulate apoptosis. These results suggest that heterogeneity in levels of gastric mucosal cell apoptosis among *H. pylori*-infected persons may result from differences in the structure and activity of VacA proteins produced by different *H. pylori* strains.

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## Induction of Gastric Epithelial Cell Apoptosis by *Helicobacter pylori* Vacuolating Cytotoxin

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