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Vacuolating Cytotoxin of *Helicobacter pylori* Induces Apoptosis in the Human Gastric Epithelial Cell Line AGS

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Helicobacter pylori **induces cell death by apoptosis. However, the apoptosis-inducing factor is still unknown. The virulence factor vacuolating cytotoxin A (VacA) is a potential candidate, and thus its role in apoptosis induction was investigated in the human gastric epithelial cell line AGS. The supernatant from the *vacA* wild-type strain P12 was able to induce apoptotic cell death, whereas the supernatant from its isogenic mutant strain P14 could not. That VacA was indeed the apoptosis-inducing factor was demonstrated further by substantial reduction of apoptosis upon treatment of AGS cells with a supernatant specifically depleted of native VacA. Furthermore, a recombinant VacA produced in *Escherichia coli* was also able to induce apoptosis in AGS cells but failed to induce cellular vacuolation. These findings demonstrate that the vacuolating cytototoxin of *H. pylori* is a bacterial factor capable of inducing apoptosis in gastric epithelial cells.**

Helicobacter pylori is a gram-negative, spiral-shaped, microaerophilic bacterium that plays a major role in the development of chronic gastritis, peptic ulcer, and gastric cancer (24, 31, 33). *H. pylori* is adapted to colonize the human stomach (24). It causes inflammation and epithelial cell damage (11), including cytoplasmic vacuolation and induction of apoptosis (40). Different virulence factors of *H. pylori*, including urease, lipopolysaccharides (LPS), adhesins, the cytotoxin-associated gene A product (CagA), and vacuolating cytotoxin A (VacA), have been described (for a review, see reference 5). About 50% of all isolated *H. pylori* strains express a functional vacuolating cytotoxin (3). The vacuolating cytotoxin is a major virulence factor in the pathogenesis of *H. pylori*-related diseases (38). The monomeric 87-kDa protein results from cleavage of a 139-kDa precursor protein (7). In bacterial cultures the monomers show the tendency to form a high-molecular-mass complex, consisting of 12 monomers with a molecular mass of about 1,000 kDa (8). VacA is suggested to exert its cytotoxic activity after internalization by epithelial cells (14).

Cell death can be executed via different mechanisms. One way is the apoptotic pathway described by Kerr et al. (18). Apoptosis is characterized by morphological and biochemical changes such as membrane blebbing, chromatin condensation, and DNA fragmentation (18). Recently, it has been shown that apoptosis is involved in *H. pylori*-induced epithelial cell damage (20, 23, 28, 34, 40, 46). It has been demonstrated that *H. pylori*-induced apoptosis is mediated via the CD95 receptor and ligand system and that this pathway is activated both from CD95 ligand (CD95L)-expressing lymphocytes and from CD95L-expressing gastric epithelial cells (40). CD95 receptor activation leads to oligomerization of CD95 receptors and to formation of a death-inducing signaling complex (19, 25, 29, 45). The death signal is transmitted via the proteolytic activation of a cascade of different caspases (for a review, see reference 36). Inoculation with *H. pylori* increases CD95L expression in epithelial cells in vitro and in the gastric epithelium in vivo (40). Nevertheless, it remains unclear which bacterial factor participates in the induction of epithelial apoptosis. Ultrafiltration of an apoptosis-inducing, cytotoxic *H. pylori* strain supernatant revealed that the apoptosis-inducing factor has a molecular mass above 300 kDa (D. Kuck et al., unpublished observation). Therefore, it was supposed that the cytotoxin VacA could be the candidate protein leading to the induction of apoptosis in gastric epithelial cells. Previous studies from Manetti et al. suggested that recombinant VacA lacks any cytotoxic activity (22). We hypothesized, however, that this lack of cytotoxic activity in their study was due to the purification of the recombinant protein under denaturing conditions. In the present study a recombinant protein was expressed and purified under native conditions, and it was able to induce apoptosis in the human gastric epithelial cell line AGS. To confirm the results obtained with recombinant VacA, the cytotoxic *H. pylori* strain P12, which expresses a functional cytotoxin, and its isogenic mutant strain P14, which possesses an inactivated *vacA* gene, were evaluated for their apoptosis-inducing properties. It was demonstrated that the supernatant of the cytotoxic strain P12 induces apoptosis, unlike the isogenic *vacA* mutant strain P14. We conclude that both recombinant and native VacA cytotoxins of *H. pylori* induce apoptosis in gastric epithelial cells.

MATERIALS AND METHODS

Bacterial culture. The following strains were used: *H. pylori* 60190 (ATCC 49503), a wild-type, cytotoxic, *cagA*-positive strain with the *vacA* genotype s1a/m1 (39); P12, a cytotoxic, *cagA*-positive strain with the *vacA* genotype s1/m1; and its isogenic mutant strain P14, which was created by transposon insertion mutagenesis (the last two were kindly provided by R. Haas, Munich, Germany). The mutagenesis of the 3′ region of the *vacA* gene was carried out via transformation of strain P12 with the plasmid pTn-73 (41). All strains were minimally passaged. A preculture was grown with shaking at 100 rpm in brucella broth containing 10% fetal calf serum (FCS) under microaerophilic conditions (10% CO_2, 5% O_2

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and 85% N_2) at 37°C. *H. pylori* medium (70% RPMI 1640, 10% FCS, 10% brain heart infusion, 10% brucella broth, 1% L-glutamine) was inoculated with the preculture, and the bacteria were cultivated for 2 to 4 days to an optical density at 600 nm of 0.5. The cultures were centrifuged at 5,000 × *g*, and the supernatants were removed and passed through a 0.2-μm filter. For VacA concentration, the supernatant was separated in a stirring cell by ultrafiltration (Centricon; Millipore, Eschborn, Germany) using a cellulose membrane (cutoff, 100 kDa; Millipore) and stored at −70°C. The supernatants were reduced 10-fold in volume by ultrafiltration.

Cell culture and cell vacuolation assay. AGS cells were cultured in Ham's F-12 medium (PAA Laboratories, Martinsried, Germany) containing 10% FCS and 1% penicillin-streptomycin (PAA Laboratories). For the cell vacuolation assay, the cells were incubated with culture supernatants of *H. pylori* for up to 24 h at 37°C in chamber slides (Lab-Tek; Nunc, Naperville, Ill.). To detect the vacuoles, cells were stained with 0.05% neutral red solution for 5 min, washed twice with phosphate-buffered saline (PBS), and analyzed by light microscopy immediately after washing.

Detection of apoptosis in AGS cells. (i) FACScan analysis. Apoptosis in AGS cells, detected by the appearance of a typical sub-G_1 fraction of fragmented nuclei, was assessed by FACScan analysis carried out in a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). Cells floating in the culture medium were collected by centrifugation at 100 × *g*. Adherent cells were harvested by incubation with 1% trypsin for 3 min. The cells were washed with PBS and fixed in 70% ethanol for less than 1 h at −20°C. After fixation, the cells were washed with PBS and stained with propidium iodide (50 μg/ml in PBS) (Sigma-Aldrich, Taufkirchen, Germany) according to the method of Nicoletti et al. (30). A minimum of 10,000 cells per sample was measured. Acquisition and analysis were conducted using Cell Quest software (Becton Dickinson).

(ii) DAPI staining and annexin V-propidium iodide staining. To identify apoptotic nuclei, DAPI (4′,6′-diamidino-2-phenylindole) (Roche-Boehringer, Mannheim, Germany) staining was performed according to the manufacturer's protocol. An annexin V-Fluos staining kit (Roche-Boehringer) was used to detect early stages of apoptosis, as represented by phosphatidylinositol flip to the outer membrane. The cells were washed with PBS and stained according to the manufacturer's protocol. Slides were mounted with Permafluor mounting medium (Immunotech, Marseille, France) and viewed under a fluorescence microscope (Axiophot Olympus).

Preparation of anti-VacA antibodies, immunoprecipitation, and Western blot analysis. (i) Preparation of rabbit anti-VacA antibodies. A DNA fragment spanning nucleotides 2317 to 2952 of the *vacA* gene of *H. pylori* strain NCTC 11638 (GenBank no. U07145) was generated by PCR using genomic DNA from *H. pylori* as template DNA. The DNA fragment was subsequently inserted into a modified pET8c vector for overexpression of the His-tagged fusion protein in *Escherichia coli*, and the bacteria were cultured at 37°C. After lysis the crude lysate was flash frozen in liquid nitrogen and thawed at 37°C. The lysate was treated with RNase at a final concentration of 5 μg/ml and with DNase at a final concentration of 5 μg/ml. The mixture was finally mixed with PBS-equilibrated ProBond resin (4:1; Invitrogen, Groningen, The Netherlands). The proteins were then eluted with PBS by column chromatography. Cleavage of the His tag was performed with enterokinase (1 U/10 μg of protein; Invitrogen). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the corresponding band was cut out, solved in PBS, and precipitated with acetone. Finally, the protein was resuspended in PBS and sterile filtered. Anti-VacA antibodies were then obtained by immunization of rabbits.

(ii) Immunoprecipitation. For immunoprecipitation of VacA, 100 μl of anti-VacA rabbit antibodies or 100 μl of control serum (preimmune rabbit serum) was incubated with 100 μl of protein A solution at 4°C on a tumbler overnight. After coupling of antibodies to protein A, the solutions were centrifuged at 3,000 × *g* and washed twice with *H. pylori* medium. The antibody-protein A pellets were resuspended in 1 ml of concentrated *H. pylori* 60190 supernatant. The bacterial supernatant was concentrated by ultrafiltration using a cutoff of >100 kDa. Depletion of VacA with anti-VacA was carried out for 4 h at 4°C on a tumbler. After centrifugation at 3,000 × *g* for 3 min, the depleted supernatants were removed, sterile filtered, and stored at −70°C. The antibody-protein A pellets were washed twice with medium and then resuspended in 100 μl of SDS sample buffer.

(iii) Western blot analysis. For protein detection, 50 μl containing supernatant and antibody-protein A pellets was loaded onto an SDS–12% polyacrylamide gel (or an SDS–15% polyacrylamide gel for detection of recombinant VacA). The proteins were transferred to a nitrocellulose membrane and washed for 20 min in washing buffer (PBS with 0.1% Tween 20). Blocking was carried out in I-Block (0.2 g of I-Block in 100 ml of washing buffer; Tropix, Perkin-Elmer, Ueberlingen, Germany) for 1 h. The anti-VacA rabbit serum was diluted 1:10,000 in washing buffer and I-Block (1:1) and incubated overnight at 4°C. After washing, the blot was incubated for 15 min with secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit; Tropix) at a dilution of 1:25,000 in washing buffer. The blot was washed for 30 min and incubated in assay buffer (9.4 ml of diethanolamine, 1 mM $MgCl_2$ [pH 10.0]) for 30 min. The blot was then incubated with Nitroblock II (1.5 ml of Nitroblock in 30 ml of assay buffer; Tropix) for 10 min, and finally, the blot was incubated for 5 min with CDP-Star (1:200 in assay buffer; Tropix) and then exposed to Hyperfilm ECL film (Amersham, Ueberlingen, Germany).

Cloning of *vacA*. For cloning of the *vacA* gene from strain NCTC 11638, PCR was carried out with the forward primer 5′ TTTGAGGCCTTTTTCACAACC GTGATCATTCCAGCC 3′ and the reverse primer 5′ TTTCTGCAGCTATTA CGAAATTTTAGAGCCATTAGCTGTTTTGCT 3′. A DNA fragment spanning nucleotides 504 to 2892 of the *vacA* gene was generated by PCR using genomic DNA from *H. pylori* as the template. The PCR product encodes amino acids 34 to 821. The first 33 amino acids of the *vacA* gene product resemble bacterial signal peptides and have been deleted.

The *E. coli* strain TOP10 (Invitrogen) was used for subcloning. The PCR fragment was subsequently inserted in the pBADHisA plasmid (Invitrogen) for overexpression. The recombinant protein had a molecular mass of 86 kDa (796 residues) without the His tag. Double-strand sequencing was carried out by the dideoxy sequencing method. Sequence alignment was carried out with the BLASTN database software tool (1).

Expression and purification of recombinant VacA. *E. coli* LMG 194 bacteria were transformed by incubation with 2 μg of DNA, and ampicillin-resistant clones were selected. For expression, a 30-ml volume was precultured overnight in RM base medium (Invitrogen) with 0.2% glucose and 100 μg of ampicillin/ml. An overnight culture with 1 liter of RM base medium containing 0.2% glucose, 100 μg of ampicillin/ml, and 25 μM dithiothreitol (Sigma-Aldrich) was inoculated at 37°C on a shaker to an optical density at 600 nm of 0.4. Protein expression was then induced with 0.0002% arabinose for 5 h. The culture was centrifuged for 30 min at 5,000 × *g*, and the cells were resuspended in PBS (pH 7.2). Egg white lysozyme was added to give a final concentration of 100 μg/ml, and the suspension was incubated for 15 min on ice. Sonication was performed with three 10-s bursts at a medium intensity while holding the suspension on ice. The crude lysate was flash frozen in liquid nitrogen and thawed at 37°C. The cells were then lysed by three successive freeze-thaw cycles. The lysate was treated with RNase at a final concentration of 5 μg/ml for 15 min at 30°C and with DNase at a final concentration of 5 μg/ml. Insoluble debris was removed by centrifugation at 3,000 × *g* for 15 min. The supernatant was mixed with PBS-equilibrated ProBond resin (4:1) (Invitrogen) and incubated on a shaker for 1 h on ice. After binding, the mixture was loaded on a column and washed with 5 volumes of PBS (pH 6.0). Elution was performed with 2 volumes of PBS (pH 4.0). The eluate was concentrated using an Amicon-10 ultrafiltration unit. Protein concentration was determined according to the method of Bradford (4).

Statistical analysis. Comparison of the data was performed using the Mann-Whitney U test.

RESULTS

Cell vacuolation assay. AGS cells were treated for 48 h with 250 μl of the concentrated supernatants/ml obtained from *H. pylori vacA* wild-type strain P12 and the isogenic *vacA* mutant strain P14. In AGS cells treated with P12 supernatant, 80% of all cells showed cellular vacuoles. In contrast, the supernatant from P14 failed to induce the formation of vacuoles (data not shown). The presence of VacA in the supernatants was confirmed by Western blotting with anti-VacA antibodies (Fig. 1a). Only the wild-type strain P12 showed a VacA signal in the range of 90 kDa by Western blotting, demonstrating that the formation of vacuoles was indeed induced by VacA.

Induction of apoptosis by *H. pylori* strains P12 and P14. Apoptosis was determined by FACScan analysis and DAPI staining. Apoptosis induction in AGS cells was observed at a minimum volume of about 100 to 150 μl of supernatant of strain P12. AGS cells incubated with 250 μl of concentrated supernatant/ml from the wild-type strain P12 for 48 h revealed in several FACScan analyses a distinct increase in cell death

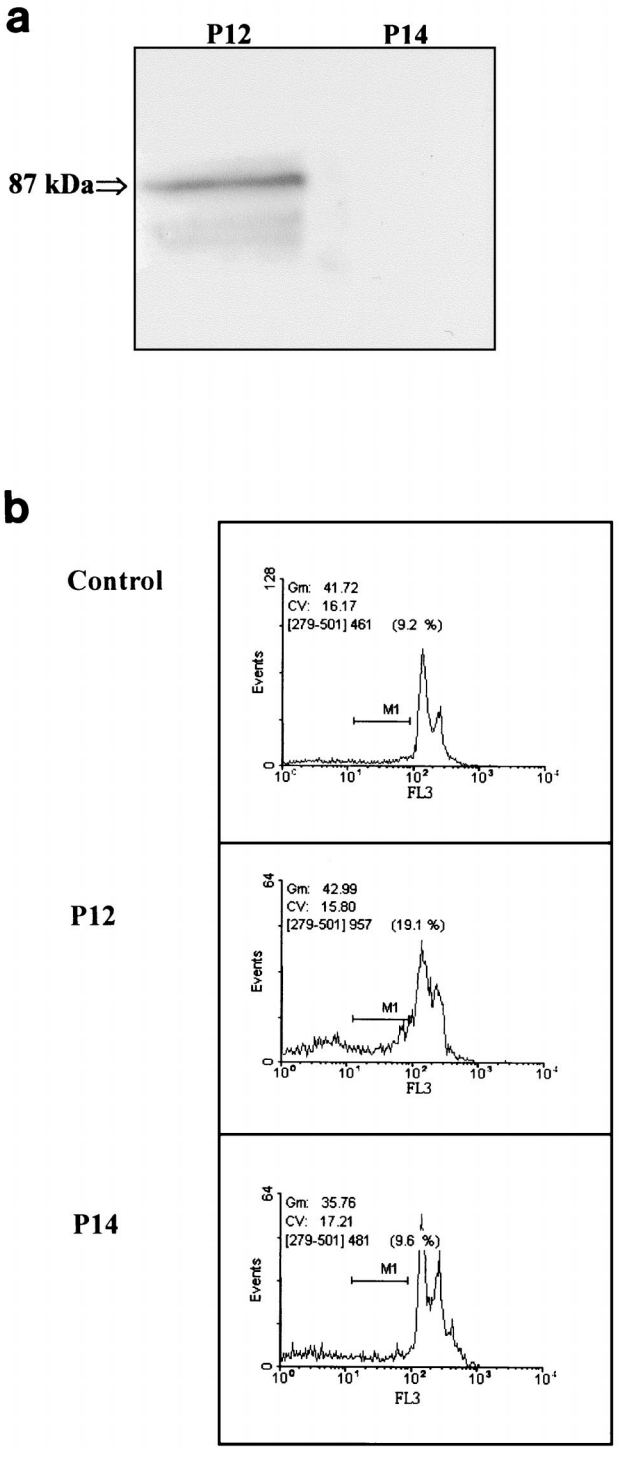

FIG. 1. (a) Western blot analysis of VacA. Fifty-microliter samples of concentrated supernatant from *H. pylori* P12 and P14 were subjected to SDS-PAGE and immunoblotted with anti-VacA antibodies. Native VacA appeared (at approximately 87 kDa) only with the supernatant of strain P12. (b) FACScan analysis of AGS cells. AGS cells were treated for 48 h with 250 μl of supernatant of *H. pylori* strains P12 and P14/ml. The abscissa displays the counts and the ordinate displays the fluorescence on a logarithmic scale. Apoptotic cells appear as a sub-G_1 peak measured through the marker region (M1). Treatment with concentrated supernatant of the *vacA* wild-type strain P12 increased the amount of apoptotic cells compared to untreated AGS cells or AGS cells treated with the isogenic *vacA* mutant strain P14.

from 7.6% ± 1.5% (mean ± standard deviation) in untreated cells to about 20.1% ± 5.9% ($P = 0.05$) in treated cells. In contrast, the concentrated supernatant from the *vacA* mutant strain P14 showed no increase in cell death (8.6% ± 1.1%, $P = 0.7$) after 48 h of incubation compared with untreated AGS cells (Fig. 1b).

Comparable results were obtained by DAPI staining of AGS cells. After 48 h of incubation with 250 μl of the concentrated supernatants from P12 and P14/ml, only incubation with supernatant from strain P12 led to the typical apoptosis hallmark of chromatin condensation. Untreated cells and cells treated with P14 supernatant presented both unchanged, normal nuclei without signs of apoptosis (Fig. 2).

Immunoprecipitation of VacA. To demonstrate that VacA is the apoptosis-inducing factor, the VacA protein was removed from the concentrated supernatant of *H. pylori* 60190 by immunoprecipitation with anti-VacA antibodies. Successful depletion was confirmed by Western blotting (Fig. 3) and by a cell vacuolation assay (not shown). Treatment of the *H. pylori* 60190 supernatant with anti-VacA antibodies led to a substantial removal of VacA from the supernatant. Control treatment with control serum (preimmune serum) revealed only a minor, nonspecific depletion of VacA from the supernatant.

Complete depletion of VacA was also confirmed in vivo by a cell vacuolation assay. AGS cells were treated for 24 h with 100 μl of supernatant/ml. The native *H. pylori* 60190 supernatant led to an extensive cellular vacuolation, whereas the vacuolation substantially decreased when AGS cells were incubated with VacA-depleted supernatant. Cells treated with control serum-depleted supernatant also showed only slight vacuole formation.

FACScan analysis of AGS cells treated with VacA-depleted supernatant. To investigate further the role of VacA in the induction of apoptosis, AGS cells were treated for 24 h with 100 μl of concentrated native and VacA-depleted supernatant/ml. The mean percentage of apoptotic cells was assessed. The percentage of apoptotic cells in untreated cells was 4.9% ± 3.2%. In cells treated with *H. pylori* 60190 supernatant, 26.0% ± 6.3% of cells underwent apoptosis ($P = 0.008$) compared to 13.4% ± 6.0% ($P = 0.05$) of cells treated with VacA-depleted supernatant and 23.5% ± 6.6% ($P = 0.008$) of cells treated with control serum-depleted supernatant. There was a significant difference in apoptosis rates between cells treated with *H. pylori* 60190 supernatant and cells treated with anti-VacA ($P = 0.008$). A trend to significance was obtained for the differences between apoptosis rates of anti-VacA-treated cells (13.4% ± 6.0%) and cells treated with control serum-depleted supernatant (23.5% ± 6.6%, P = 0.095).

Recombinant VacA induces apoptosis in AGS cells. To confirm the apoptosis-inducing activity of recombinant VacA, we expressed the recombinant cytotoxin in *E. coli* LMG 194 using the pBADHis system. Recombinant VacA was analyzed by Coomassie staining (data not shown) and by Western blotting with anti-VacA antibodies (Fig. 4). The recombinant protein, composed of an 86-kDa VacA part corresponding to the sequence of the native soluble VacA and an N-terminal 3-kDa histidine tag, showed the expected molecular mass of about 90 kDa by Western blot analysis. Double-strand sequencing of the plasmid revealed three point mutations in the amino terminus-coding region when compared with the native *vacA* sequence

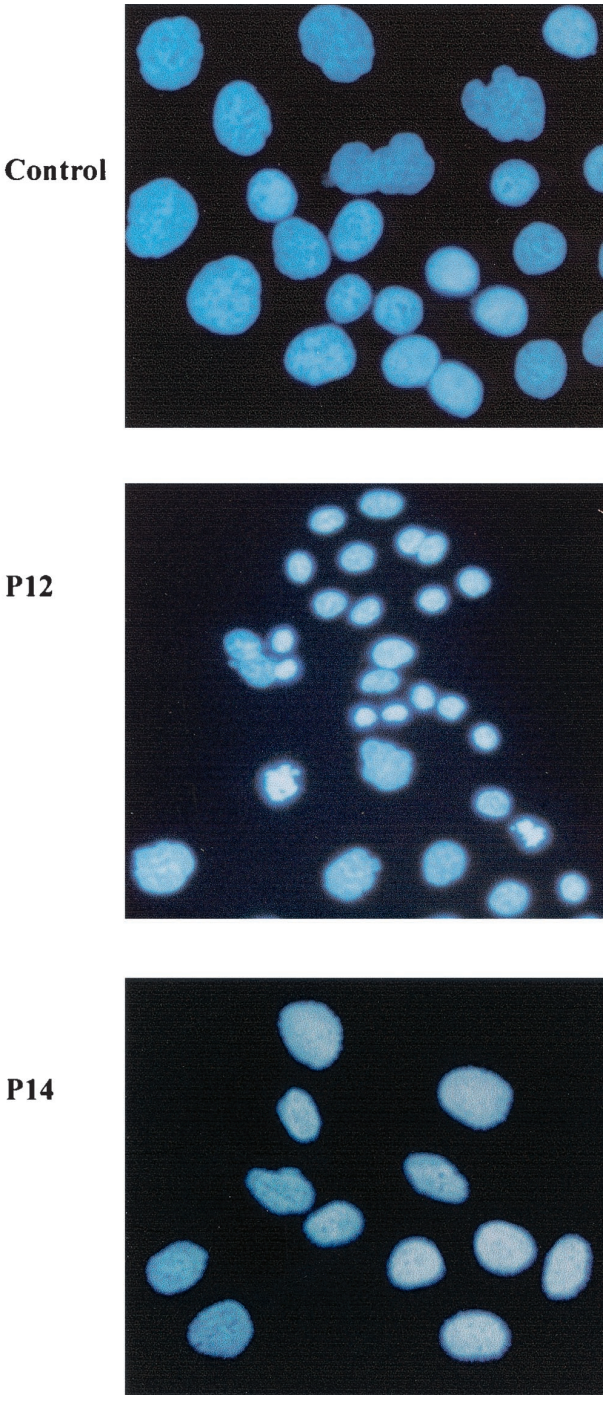

FIG. 2. DAPI staining of AGS. AGS cells were incubated for 48 h with 250 μl of supernatants from strains P12 and P14/ml. Nuclei are stained blue. Incubation with supernatant of the *vacA* wild-type strain P12 increased the amount of apoptotic cells, which are characterized by chromatin condensation with brighter fluorescence. In contrast, the supernatant from its isogenic *vacA* mutant strain P14 showed only normal, noncondensed nuclei. Magnification, ×400.

of strain NCTC 11638. The mutations led to exchanges of residues 68 (Tyr→His), 149 (Ala→Thr), and 231 (Tyr→His). To detect apoptosis, AGS cells were incubated with 100 μg of recombinant VacA/ml for 60 h. FACScan analysis demonstrated a distinct increase in apoptotic cell death in the range of 19.9% ± 2.1% in treated cells compared to 8.2% ± 2.5% ($P = 0.05$) in untreated cells (Fig. 5). Additionally, after 60 h of incubation, a remarkable increase of apoptotic, condensed nuclei was observed by DAPI staining (Fig. 6). Similar findings were obtained with annexin V-propidium iodide staining, which showed an increase in apoptotic cells (Fig. 6). The minimum concentration of recombinant VacA for apoptosis induction was in the range of 50 to 100 μg/ml. However, recombinant VacA failed to induce cellular vacuolation (data not shown).

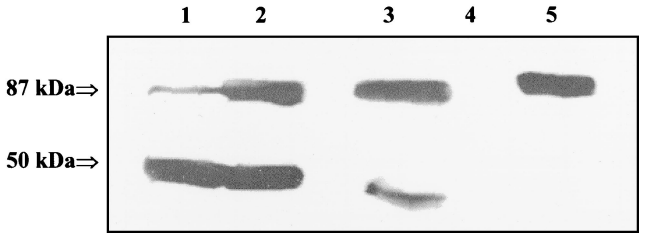

FIG. 3. Western blot analysis of immunodepleted and concentrated supernatants of *H. pylori* 60190. The antibody (anti-VacA antibodies or control serum)-protein A pellets and the depleted supernatants were subjected to SDS–12% PAGE and immunoblotted with anti-VacA antibodies. Lane 1, precipitated pellets with control serum; lane 2, precipitated pellets with anti-VacA antibodies; lane 3, supernatant depleted with control serum; lane 4, supernatant depleted with anti-VacA antibodies; lane 5, native *H. pylori* 60190 supernatant. The 87-kDa band represents VacA, and the 50-kDa band represents the heavy chain of Igs. Incubation with anti-VacA antibodies coupled to protein A substantially removed VacA from the *H. pylori* 60190 supernatant (lanes 2 and 4). Precipitation with control antibodies demonstrated only a slight precipitation of VacA (lanes 1 and 3).

DISCUSSION

Apoptosis plays a major role in the pathogenic action of *H. pylori* (20, 23, 28, 34, 40, 46). The CD95 receptor and ligand system was previously identified as a mediator of apoptosis induction in gastric epithelial cells in vitro and in patients with *H. pylori*-induced chronic gastritis in vivo (40). However, it was still unclear which bacterial factors of *H. pylori* are involved in apoptosis induction. The results of the present study demonstrate that VacA is at least one of the *H. pylori* factors capable of apoptosis induction.

First, it was shown by FACScan analysis and DAPI staining that the supernatant of the cytotoxic wild-type *H. pylori* strain P12 induces apoptosis in AGS cells. In contrast, the supernatant from its isogenic *vacA* mutant strain P14 has lost its ap-

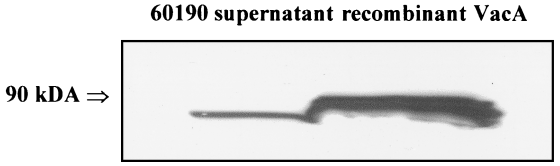

FIG. 4. Western blot analysis of recombinant VacA. Twenty-five micrograms of recombinant protein and 50 μl of concentrated *H. pylori* 60190 supernatant were subjected to SDS–15% PAGE and immunoblotted with anti-VacA antibodies. Native VacA appeared at nearly 87 kDa, and the recombinant VacA appeared at 90 kDa.

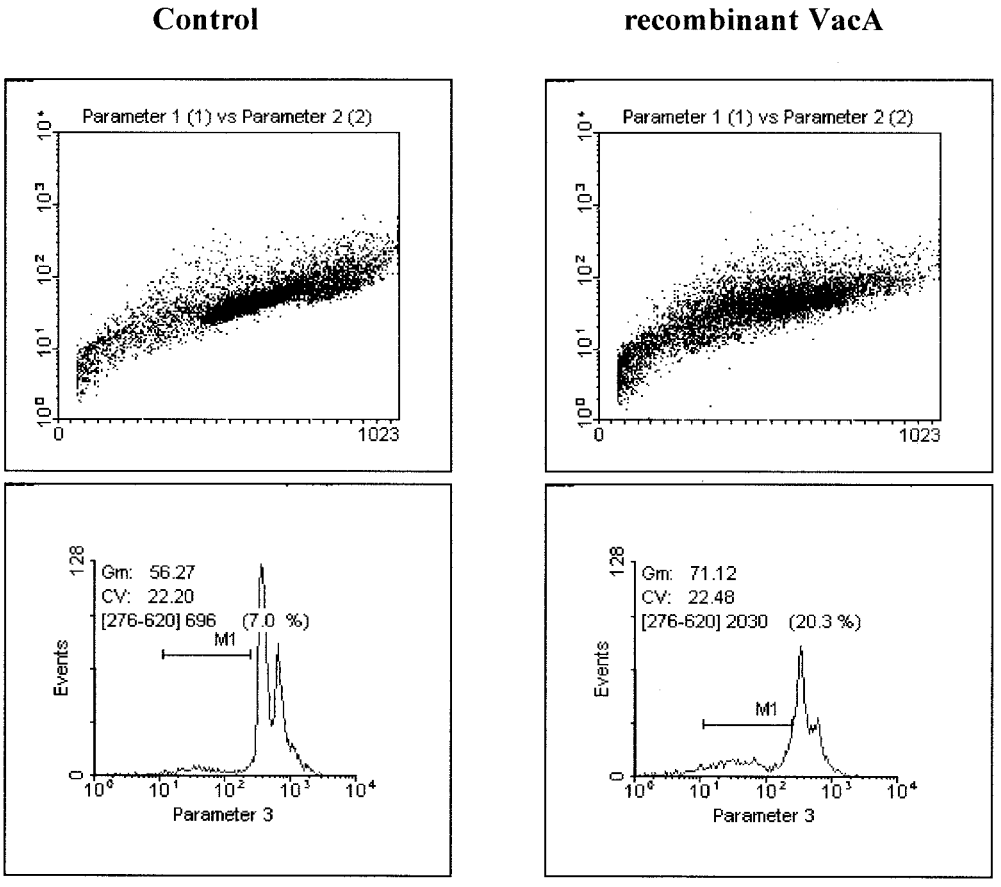

FIG. 5. FACScan analysis of AGS cells. AGS cells were incubated for 60 h with 100 μg of recombinant VacA/ml. The abscissa displays the counts, and the ordinate displays the fluorescence on a logarithmic scale. Apoptotic cells appear as a sub-G_1 peak, which was statistically acquired through the marker region (M1). Incubation with recombinant VacA increases apoptotic cell death (right).

optosis-inducing activity. Further confirmation was provided by immunodepletion of native VacA with anti-VacA antibodies from the supernatant of *H. pylori* 60190. Quantitative removal of VacA from the supernatant after immunoprecipitation was confirmed by cell vacuolation assay and by Western blotting with anti-VacA antibodies. Depletion of VacA led to a substantial reduction of vacuole formation. Analysis of AGS cells treated with VacA-depleted supernatant demonstrated a reduced induction of apoptosis, compared to the apoptosis induction by native, nondepleted *H. pylori* 60190 supernatant. Control immunoglobulin (Ig)-depleted supernatant showed only a slight reduction of apoptotic activity due to unspecific binding of VacA to Ig. These results strongly indicate that VacA is a bacterial factor capable of inducing apoptosis.

In contrast to the present results, apoptosis could not be induced with lower concentrations of VacA (10 μg/ml) in a recent study (35). This may be due to larger amounts of VacA used in the present study, with estimated VacA concentrations of about 200 μg/ml in 10-fold-concentrated supernatant.

To further confirm the role of VacA in the induction of apoptosis, recombinant VacA was generated using an expression system consisting of the vector pBADHis and the *E. coli* strain LMG 194 (15, 16). Using this system, a recombinant protein that induces apoptosis in the human gastric epithelial cell line AGS was expressed and purified under native conditions. FACScan analysis of AGS cells after incubation with recombinant VacA revealed a substantial induction of apoptosis. In addition, the apoptosis-inducing activity of recombinant VacA was confirmed by DAPI staining and annexin V-propidium iodide staining of AGS cells incubated with recombinant VacA. The recombinant protein in this system was not, however, able to induce vacuole formation. Double-strand sequencing of the plasmid revealed three mutations in the amino terminus coding region. These amino acid exchanges may be responsible for incomplete protein folding during expression, causing the protein to lose its vacuolating activity. Recently, it has been shown that the vacuolating activity of VacA may be confined to the amino-terminal domain of VacA (9). Previous attempts to express a recombinant VacA resulted in a protein which completely lacked any biological activity (22). This may be due to inclusion bodies formed during protein overexpression. The presence of inclusion bodies requires the protein to be purified under denaturating conditions and subsequently refolded. To avoid incomplete or incorrect folding, an expression system for VacA which allows the generation of the protein without overexpression was used (15, 16). A functional active recombinant VacA with apoptosis-inducing properties could be generated using this system.

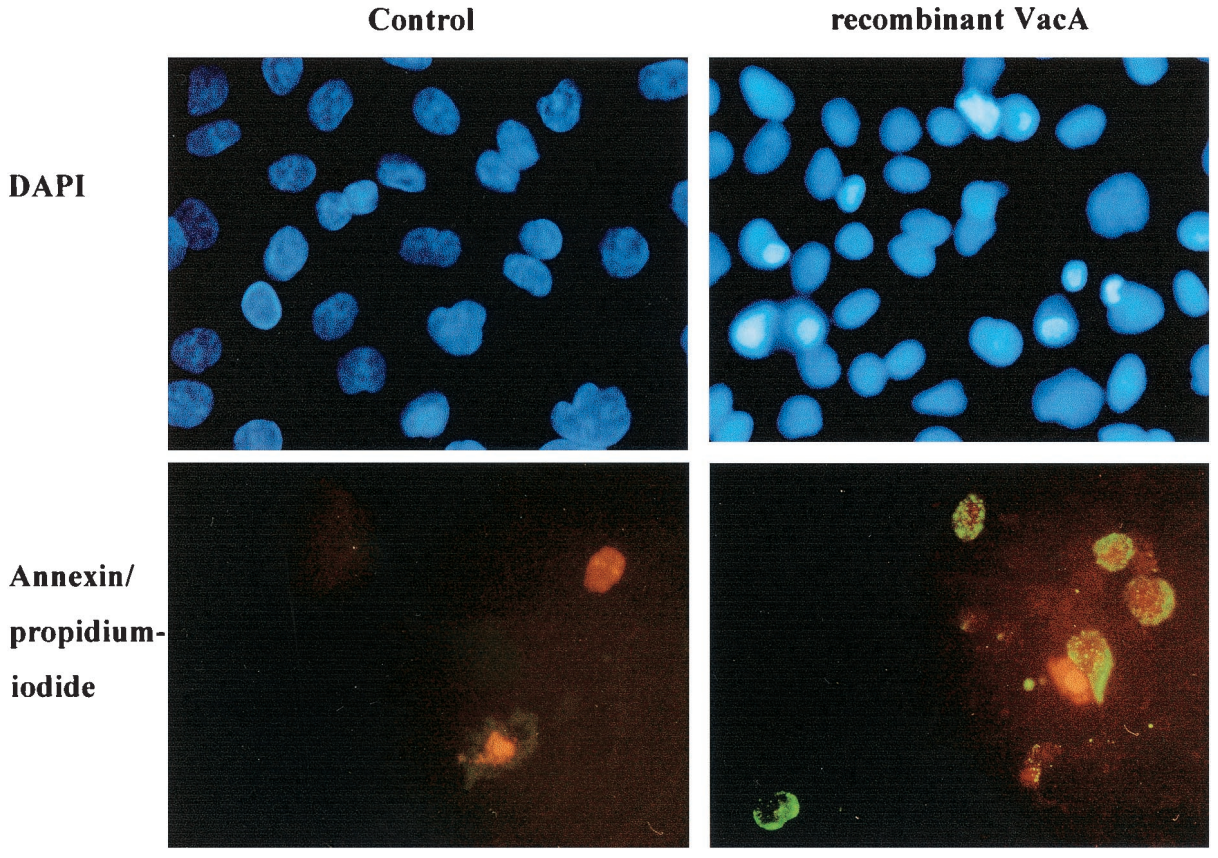

FIG. 6. DAPI staining and annexin V-propidium iodide staining of AGS cells. AGS cells were treated for 60 h with 100 μg of recombinant VacA/ml. DAPI-stained nuclei are blue. Annexin V-stained cells appear green, which characterizes the early stage of the apoptotic process. Propidium iodide labels the necrotic cells red. After incubation with recombinant VacA, DAPI-stained nuclei showed apototic, condensed chromatin (top right) and annexin V-propidium iodide-stained cells showed signs of early apoptotic stages (bottom right). Untreated cells demonstrated normal nuclei with DAPI staining (top left) and no apoptosis induction with annexin V-propidium iodide staining (bottom left). Magnification, ×400.

However, VacA might exert apoptosis induction and vacuolating cytotoxicity through different domains and by different mechanisms, which could explain the presence of induction of apoptosis and the lack of vacuolating activity. The mechanism of cell vacuolation by VacA has been partly characterized. It has been hypothesized that VacA assembles at an acidic pH into a hexameric anion-selective channel in planar phospholipid bilayers (8, 10, 17, 21). After binding to the cell surface, the toxin translocates into the cytosol, where it may affect a key molecule involved in the control of membrane trafficking of late endosomes and lysosomes. VacA can assemble to an ion channel in the vacuolar membrane, which at least in part is responsible for vacuolar swelling (26, 27). In contrast, the way VacA induces apoptosis is still unclear. A proposed mechanism of apoptosis induction by VacA is the activation of the CD95 receptor and ligand system (40). Microinjection of DNA encoding VacA-GFP (green fluorescent protein) into HEP-2 cells induced apoptotic cell death in a very recent study (13). It was further shown that the N-terminal fragment of VacA (p34) induces cytochrome *c* release and apoptosis (13).

The results, however, indicate that bacterial factors of *H. pylori* other than VacA may be involved. Immunodepletion of VacA did not result in total disappearance of the apoptosis-inducing capacity from the supernatant as was expected based on the results of Western blotting and cell vacuolation assays. This may also be explained by differences in the limits of detection of these methods, and several other factors, such as LPS (37), urease (20), and the *picB* product (35), have been suggested to contribute to *H. pylori*-mediated apoptosis. Isolated LPS from a cytotoxic *H. pylori* strain increased the rate of apoptosis throughout the gastric epithilium in rats (37). In addition, a significant correlation between urease activity and the level of apoptosis in the mucosa of infected patients with active ulcer disease has been reported (20). *H. pylori* protease and lipase degrade gastric mucus and disrupt the phospholipid layer of the cells, which may lead to apoptotic cell death (43). Another factor contributing to apoptosis induction could be CagA. Recently, several investigators have shown that CagA is delivered into epithelial cells by the *cag* type IV secretion system and is subsequently phosphorylated (2, 32, 44). The majority of analyzed *cagA*$^+$ strains induced enhanced apoptosis when compared to *cagA*-negative strains (35). Other investigators could not confirm these findings (42). The pres-

ence of other possible apoptosis-inducing factors might, in part, explain why some investigators could not find a significant difference in apoptosis induction between VacA-positive and VacA-negative strains (46).

This study provides evidence that the cytotoxin VacA is a bacterial factor capable of inducing apoptosis in AGS cells. It was shown for the first time that both native and recombinant VacA have apoptosis-inducing activity. VacA-producing *H. pylori* strains are found in the vast majority of patients with peptic ulcer disease (PUD) (6, 12). VacA has been shown to be directly involved in mucosal damage in PUD (43). This damage might be attributed to the vacuolating as well as to the apoptosis-inducing activity of VacA. Therefore, elucidation of VacA as an apoptosis-inducing factor might offer a better understanding of the pathogenesis of *H. pylori*-related diseases such as PUD.

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