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Pathogenetic Impact of Vacuolar Degeneration by Accelerated Transport of *Helicobacter pylori* VacA

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Abstract Vacuolar degeneration of the gastric epithelial cells is a characteristic feature of the derangement of mucosa where *Helicobacter pylori* colonizes, and *H. pylori* vacuolating cytotoxin (VacA) has been suggested to play a key role in it. To elucidate the VacA-involved degenerative mechanism, VacA was purified, and its impact on degeneration of HeLa cells was determined. In the presence of ammonium chloride, cell vacuolation by VacA was dose- and time-dependent, however, no detectable degeneration of the cells was observed with the VacA concentration tested. A further increase of vacuolation was shown in cells pre-treated with diethyl pyrocarbonate (DEPC), and this resulted in a change of the cell morphology to become spherical. Similar phenomena were also observed when HeLa cells were co-cultivated with intact *H. pylori* cells. It was remarkable to note that the degree of growth inhibition was proportional to the increase in vacuole formation, suggesting that the vacuolation rate would be critical for cell degeneration. Surprisingly, although VacA was itself inhibited by DEPC, its uptake was markedly increased by this agent, similar to that found in cells with Na-butyrate. These data indicate that the cell's tolerance of VacA transport may be critical for vacuolar degeneration and may be changeable during *H. pylori* inhabitation.

Key words: *Helicobacter pylori*, VacA cytotoxin, VacA transport, vacuolar degeneration

Although some closely related bacteria appear to be present in primates, *Helicobacter pylori* is the only bacterium known to inhabit the human stomach [11]. It is now well established that this bacterium is associated with occurrence and recurrence of chronic and acute gastritis and peptic ulcer disease, and it is the major risk factor in the development of gastric cancer [23, 24]. Despite persistent infection by *H. pylori*, however, the probability of a deleterious outcome with severe complications is extremely low; in most cases, *H. pylori* inhabits without significant detriment to humans [8]. For this reason, generalized mechanisms of pathogenesis attributable to *H. pylori* infection have yet to be established. Instead, individual roles by the constitutive active virulence factors have been proposed, such as urease [10, 16], γ-glutamyltranspeptidase [3, 19], mucinase, motility, or vacuolating cytotoxin (VacA) with an immunodominant protein (CagA) [4]. On the other hand, histological evidences obtained from biopsies of stomach epithelium near *H. pylori* colonies suggest a close relationship between pathogenesis and mucosal derangement [9, 31].

Gastric mucosa is known to resist injury, as it is capable of rapidly repairing the damaged area using adjacent tissue, perhaps by passive migration of mucous neck cells. The resultant denuded epithelial gap can readily be healed by virtue of the active proliferation of cells from the gastric stem gland [12, 29]. This, together with the above-mentioned significant clinical disparity with *H. pylori*-infected persons, suggest that the gastric pathogenesis of *H. pylori* is opportunistic, resulting in disruption of the mucosal defense process *per se*. In gastritis patients, epithelial cells are often extensively vacuolated, resembling those found in germ-free piglets experimentally infected with *H. pylori* [22, 27]. Unnatural vacuolation proceeds with no known reason, but it appears that the extent delimits the cell's life-span [2]. Accordingly, increasing the rate may be injurious to tissue, causing tissue depletion like the symptomatic gastric mucus in which *H. pylori* colonizes. Meanwhile, it has empirically been observed that the incidental release of gastric acid in response to sympathetic stress is partly associated with acute stomach disease. Interestingly, both expression and cytotoxic activity of *H. pylori* VacA are highly stimulated under the acidic conditions, which suggests cooperation of gastric acid and VacA in the pathogenesis of *H. pylori* [6]. However, normally in the acidic gastric lumen, the protective mucus layer allows gastric surface

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epithelium to maintain neutrality. Therefore, unless the natural potential of the mucus repair system were perturbed, such cooperation may not occur. In fact, it has been reported that the relative prevalence of VacA produced in patients with gastritis or peptic ulcer disease is inconsistent, although most *H. pylori* isolates hybridize with *vac*A probe [25].

In view of these considerations, we suspected whether VacA could exert vacuolar degeneration *in vivo*, especially under acid-free conditions. In order to verify the essential nature of VacA, we attempted to detect VacA-caused cell degeneration and found that fatally degenerative vacuolation could occur even with small quantities of *H. pylori* VacA, incidentally with rapid vacuolation.

MATERIALS AND METHODS

Culture Conditions

HeLa cells were seeded in Eagle's modified minimum essential medium supplemented with 10% fetal bovine serum in 96-well titration plates (1×10^4 cells per well) and cultured in the presence or absence of an appropriate concentration of either VacA and/or DEPC for 2 days in a 5% CO_2 incubator at 37°C [4]. Viable cells were counted by the trypan blue exclusion test. *H. pylori* ATCC 49503 was grown for 2 days at 37°C on agar plates containing a brain heart infusion medium (Difco; pH 6.8) supplemented with 5% virus-free horse serum (GibcoBRL Life Technology, U.S.A.) in a 10% CO_2 humidified incubator [33].

To prepare intact HeLa cells, cells were harvested by treatment with trypsin, washed twice by resuspending into a cold phosphate-buffered saline solution (pH 7.4), and centrifuged for 10 min at 1,800 ×g. The washed cells were then suspended to give 1×10^5 to 10^6 cells/ml into 20 mM N'-2-hydroxypiperazine-N-2-ethanesulfonate (HEPES; pH 7.4) containing 2% D-glucose, 1 mM [ethylenedinitrilo] tetraacetate (EDTA), and 2 mM $MgCl_2$, which was used for transport assay.

Purification of *H. pylori* VacA

VacA toxin protein was purified from the *H. pylori* culture broth. In practice, 750 ml of the culture filtrate (total vacuolating O.D. at 540 nm was about 400; ca. 0.5 U of VacA) was concentrated and fractionated by ammonium sulfate, followed by consecutive column chromatography with DEAE-Sepharose CL-6B and Sephacryl S-300, as described previously [4]. By this procedure, about 10% of VacA was recovered from the *H. pylori* culture filtrate, which possessed 10- 15 U of vacuolation activity.

Assay of Vacuolating Activity

At the beginning of the cell cultivation, either thoroughly washed *H. pylori* cells (1×10^4 to 10^5 cells per well) or purified VacA sample (ca. ~0.5 U) were added and incubated in the presence or absence of diethyl pyrocarbonate (DEPC). Culture supernatant of *H. pylori*, passed through a 0.45-μm filter unit (Millex-HV; Millipore, Bedford, MA, U.S.A.), was separately tested as a control. In all cases, ammonium chloride was added to a final 10 mM concentration to induce vacuolation. Visible vacuolation was easily assessed microscopically at 200-fold magnification. Vacuolating activity was determined spectrophotometrically at 540 nm by measuring the amount of neutral red taken up by acidic vacuoles; an acidic ethanol (pH 3.0, adjusted by 0.1 N HCl) was used to extract the dye. One unit (U) of VacA was defined as 1.0 absorbance unit at 540 nm per milligram of proteins. The data shown are means ± standard errors of triplicate experiments.

Transport Assay

Peptide: L-phenylalanyl-L-3-thiaphenylalanine (PSP) at 0.1 mM final concentration was added to the intact HeLa cell suspension containing 0.1 mM 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), and the suspension was then incubated at 37°C. Aliquots were taken during incubation, centrifuged to remove cell pellets, and the absorbance of the yellow adduct present in the supernatants was measured spectrophotometrically at 412 nm. To ascertain any leakage from the plasma membrane by DEPC, portions of the medium were taken by centrifugation of the cells that were pre-treated with DEPC, and were reacted directly with the above detector, PSP, in the presence of DTNB. In all cases, samples placed on an ice-bath were used as controls.

Vac A protein: Purified Vac A proteins treated or untreated with DEPC or Na-butyrate were dialyzed against 20 mM HEPES buffer (pH 7.4) overnight at 4°C. Then, the dialyzed samples were added to the intact HeLa cells suspension, followed by incubation at 37°C, and the amounts remaining in the medium after centrifugation were quantified. The protein concentrations were estimated using a Bradford assay kit (Sigma; St. Louis, U.S.A.).

RESULTS

Vacuolating Properties of *H. pylori* VacA

H. pylori VacA protein aggregates irregularly in aqueous compartments, but is capable of membrane spanning, whereupon it functions like a V-type ATPase [5]. Nevertheless, the vacuolation is believed to be uncontrollable, because VacA-mediated vacuolation kinetics should be conditional due to its xenobiotic nature. Therefore, vacuolar degeneration would appear to be opportunistic.

To demonstrate this, we investigated the degenerative mechanism using a purified VacA sample. As predicted, the cell vacuolation increased proportionally with the increase in the VacA concentration (Fig. 1A), however, the relative

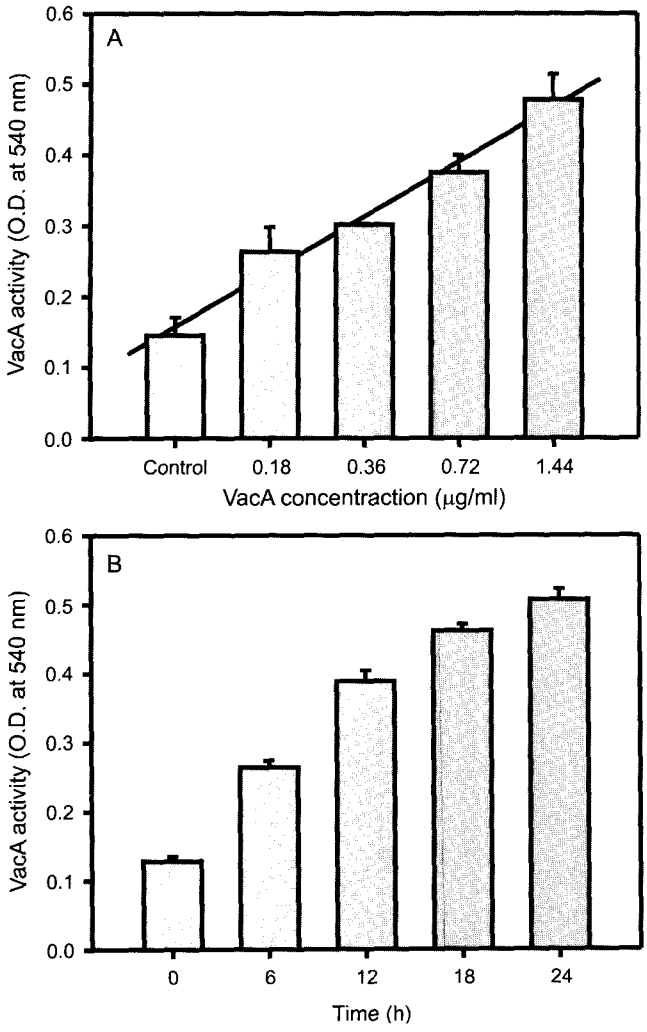

Fig. 1. Determination of vacuolating property of *H. pylori* VacA in HeLa cells.

A, Effect of VacA concentration on cell vacuolation. Culture medium containing a purified VacA sample ranging 0.18- 1.44 μg/ml and 10 mM NH_4Cl was added to each well with HeLa cells (1×10^4 cells/ml), and incubated for 2 days. VacA activity was assayed by measuring the amount of neutral red extracted with an acidic ethanol, and expressed as optical density as described in Materials and Methods. Cells incubated with only ammonium chloride were used as a control. B, Aging dependence of VacA activity. A given quantity of VacA and ammonium chloride were consecutively added to each of the HeLa cell cultures at a different culture time. During cultivation of HeLa cells, time-course addition of VacA was separately made to each culture, with a time-interval of 6 h. After 6 h of cultivation upon last addition, the resultant extent of vacuolation in each culture was measured and the values were plotted as a function of the addition time. The further incubation time with VacA to meet 2 days is presented in the horizontal axis. Data are the means of triplicate assays, and bars represent ± S.D.

extent changed apparently with the cells aging; an estimated increase of about 2-fold for every 6 h of the cultivation time, giving a typical hyperbola (Fig. 1B). A similar phenomenon was also observed in a stomach cancer cell-line (data not shown), indicating that VacA-mediated vacuolation might have a so-called conditional kinetics to take place. Nonetheless,

Table 1. Determination of PSP transport in HeLa cells pre-treated with VacA or DEPC.

VacA		DEPC	
Pre-incubated time (h)	Thiophenol released (nmoles/min)[a]	Conc. (mM)	Thiophenol released (nmoles/min)
0	1.5±0.1	0	1.4±0.1
6	1.7±0.1	0.1	1.4±0.2
12	1.6±0.2	0.2	1.4±0.2
18	1.5±0.2	1	1.2±0.3
24	1.3±0.3	2	1.2±0.3

[a]Mean values of the amounts of thiophenol released after intracellular hydrolysis of PSP during 20 min of transport in HeLa cells of 1×10^6 cells/ml. The rate was computed according to the molar extinction coefficient at 412 nm (13,600 $M^{-1}cm^{-1}$).

all the vacuolated cells were still viable without showing any detectable degeneration (see Fig. 6B), since their ability to assimilate small peptides was unchanged (Table 1).

Relationship Between the Vacuolating Rate and the Cell's Degeneration

To increase the degree of vacuolation, HeLa cells were treated with DEPC, a histidine- or tyrosine-modifying agent [30], and then incubated with VacA. In fact, DEPC by itself appeared to promote the cell's vacuolation and growth inhibition to some extent, but not significantly (Fig. 2). Unexpectedly, this agent was shown to inhibit VacA activity in proportion to the concentration. As can be seen in Table 2, VacA was activated by acid or alkaline treatment, but DEPC treatment did not restore the activity, regardless of dialysis employed, perhaps indicating an irreversible inactivation involving structural alteration. In contrast, further increase of VacA activity towards the cells

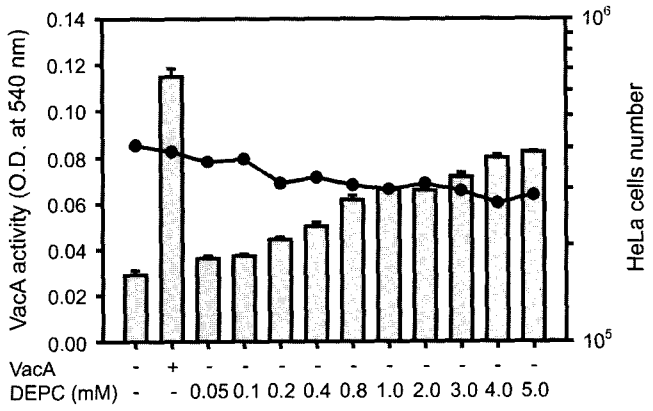

Fig. 2. Vacuolating property of DEPC.

HeLa cells were grown for 2 days in the presence of different DEPC concentrations as indicated and 10 mM NH_4Cl. For comparison, data values with VacA plus ammonium chloride are presented. For assay conditions and data expression, see the legend of Fig. 1. For counting of viable cells (●), see Materials and Methods.

Table 2. Effect of DEPC on VacA activity in HeLa cells.

(A) Effect of DEPC treatment on acid-activation of VacA

DEPC conc.(mM)	VacA activity (U)		
	1st at pH 7.0	2nd at pH 2.0	Activation degree*
0	0.27±0.01	0.42±0.02	1.56
0.1	0.24±0.01	0.26±0.03	1.08
0.2	0.16±0.03	0.22±0.04	1.38
1	0.13±0.02	0.11±0.02	0.85
2	0.11±0.02	0.07±0.02	0.64

*Relative ratio of the vacuolating activity of *H. pylori* VacA treated with a low pH condition (pH 2.0; 37°C for 1 h) to the beginning one (pH 7.0).

(B) Recovery of VacA activity after DEPC treatment

pH Change		DEPC treatment	Remained VacA activity (U) after dialysis at pH 7.0
From	To		
2	7	-	0.58±0.04
2	7	+	0.23±0.04
7	7	-	0.38±0.01
7	7	+	0.18±0.03
7	2	+	0.20±0.02
7	10	+	0.22±0.02
10	7	-	0.51±0.02
10	7	+	0.22±0.04

VacA was dissoved in a 5 mM phosphate-buffered saline solution, pH adjusted by 0.1 N HCl or NaCl, and then incubated in the presence (+) or absence (-) of 1 mM DEPC for 1 h at 37°C. To the resulting solutions were added 50 mM Na-phosphate/-borate with a given pH in excess (e.g., 1:10, v/v), then incubated again as above. Dialysis was done against 50 mM Tris-HCl (pH 7.0) for overnight at 4°C, using Dialysis tubing (Sigma; Mr 12,000 limit).

pre-treated with DEPC was shown with Na-butyrate. Unlike the former, the latter did not appear to inhibit VacA (Table 3); Substantial toxicities were found, especially over 1 mM DEPC, where reverse relationship between the rates of cell vacuolation and growth was found (Fig. 3). Moreover, the resultant cells appeared to be exclusively spherical, implying an impact of the vacuolation rate on cell degeneration (Fig. 6C).

Using L-phenylalaninyl-L-3-thiaphenylalanine (PSP) [17] at up to 2 mM of DEPC concentration, HeLa cells did not show any functional abnormity in the plasma membrane,

Table 3. Effect of Na-butyrate on VacA activity in HeLa cells.

Na-butyrate Conc. (mM)	VacA activity (U) after treatment only against	
	VacA	HeLa cells
0	0.37±0.02	0.37±0.02
1	0.36±0.01	0.62±0.05
2	0.38±0.04	0.73±0.08
4	0.39±0.03	0.69±0.03

Note that significant increase in vacuolation is seen only in the case of HeLa cells, but not of VacA, of those preteated with Na-butyrate.

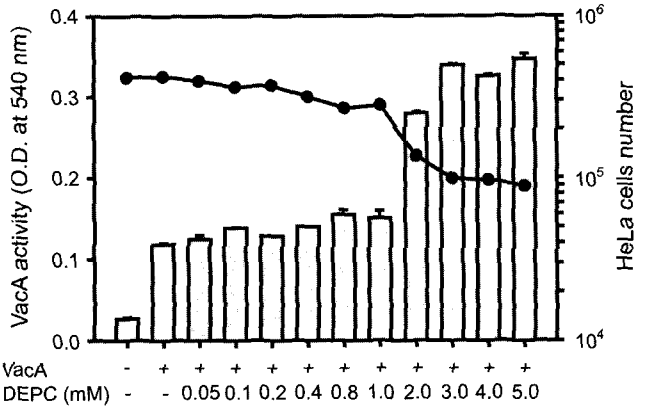

Fig. 3. Effect of DEPC on VacA-mediated vacuolation.
HeLa cells were cultivated for 2 days in the presence of VacA and DEPC at the concentration indicated. For detailed conditions, refer to the above figures. Note that the right-handed vertical values for cell numbers (●) differ from that of Fig. 2.

where the above detector peptide was normally transported intact into the DEPC-treated cells without significant change in the rate (see Table 1). Also, no peptidase leakage was detected in the transport system during the experiment (data not shown). These data suggest that DEPC permits the cell's fundamental function to proceed, especially nutritional intake, thus implying viability (see Table 1). On the other hand, VacA transport in the DEPC-treated cells was rather enhanced; although the transport rate of the VacA treated with DEPC decreased, DEPC allowed actual VacA transport to be increased (Fig. 4A).

A similar increase of VacA transport was also shown with Na-butyrate, even though Na-butyrate itself did not directly affect VacA (Fig. 4B). Despite the apparent difference between DEPC and Na-butyrate in their effects on VacA activity, they resemble each other in that their action to increase both VacA activity and transport. Therefore, VacA activity, even if partly, appears to be closely dependent on its uptake rate. Based on this supposition, VacA-mediated vacuolation might involve a committed step at the level of the plasma membrane, where the rate of VacA transport would be regulated.

Effect of Co-cultivation with Intact *H. pylori* Cells

To obtain further evidence about vacuolar degeneration, an experiment was undertaken using intact *H. pylori* cells. HeLa cells were co-cultivated with a range of *H. pylori* concentrations, and the extent of cell vacuolation and viability was determined. When results obtained were plotted as the function of initial doses of *H. pylori* cells, they showed a sequence of exponential increases in the degree of vacuolation with decreasing cell growth (Fig. 5): Over 6-fold relative ratio of initial *H. pylori* dose to that of HeLa cells, the relation between the extent of vacuolation and cell growth was inversely proportional, in concordance

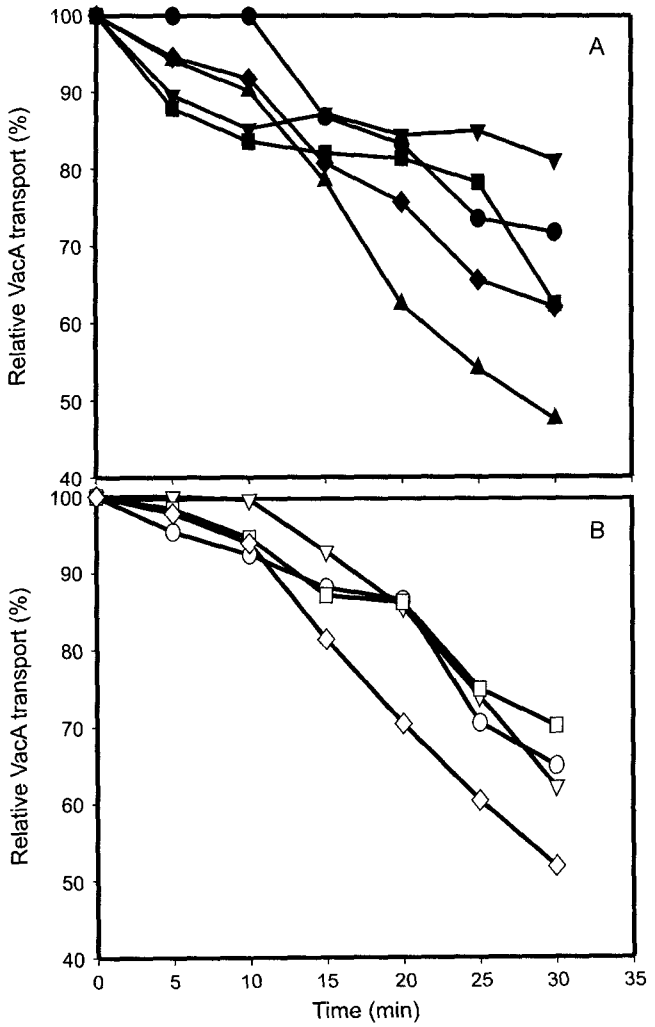

Fig. 4. Effect of DEPC and Na-buyrate on VacA transport in HeLa cells.
A, DEPC; B, Na-buyrate. Symbols used: ●, VacA only; ▼, VacA treated with 1 mM DEPC; ■, after dialysis of the DEPC-treated VacA; ◆, HeLa cells pre-treated with 1 mM DEPC; ▲ for with 2 mM DEPC; ○, VacA only; ▽, VacA treated with 2 mM Na-butyrate; □, VacA transport in the presence of 2 mM Na-butyrate; ◇, HeLa cells pre-treated with 2 mM Na-butyrate.

with the data of Fig. 3. Under such situation, *H. pylori* colonized well and seemed to cause severe toxicity reducing cell densities of HeLa cells, accompanied also by spherical morphology (Fig. 6D). Taken together with the finding with DEPC, this suggested that *H. pylori* produced some factor that mobilized the host cell's membrane, thus enabling VacA to be easily taken up. In fact, our preliminary study, using *H. pylori* culture filtrate, strongly suggests that *H. pylori* secretes a factor with a low molecular weight, which is dialyzable (data not shown). In any case, circumstantial evidence indicates that the VacA-mediated vacuolation rate can be modulated, and it is changeable depending on the extent of *H. pylori* infection.

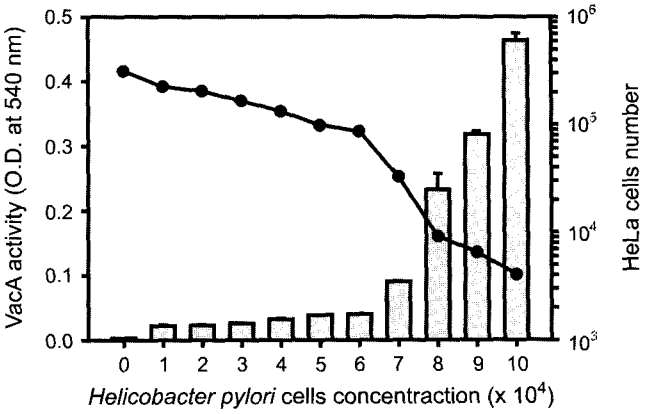

Fig. 5. Cytotoxic effect of intact *H. pylori* cells.
To HeLa cells with an initial concentration of 1×10^4 per milliliter, was added *H. pylori* cells as indicated, followed by co-cultivation with 10 mM of initial concentration of ammonium chloride for 2 days in a 5% CO_2 incubator at 37°C. For assay conditions and data expression, see above figures. Note vertical values for viable cell numbers (●).

DISCUSSION

H. pylori VacA is known to be activated permanently under both acid and alkali conditions [32]. The toxin is also activated upon treatment with certain proteases like pepsin, revealing a unique structural versatility. The amino-terminal sequence of VacA was reported to be partly homologous to various ion channels or membrane permeases of eukaryotes, unlike other bacterial toxins targeting intracellularly. On the other hand, membrane trafficking is supposed to be important in VacA uptake, because the process is mediated

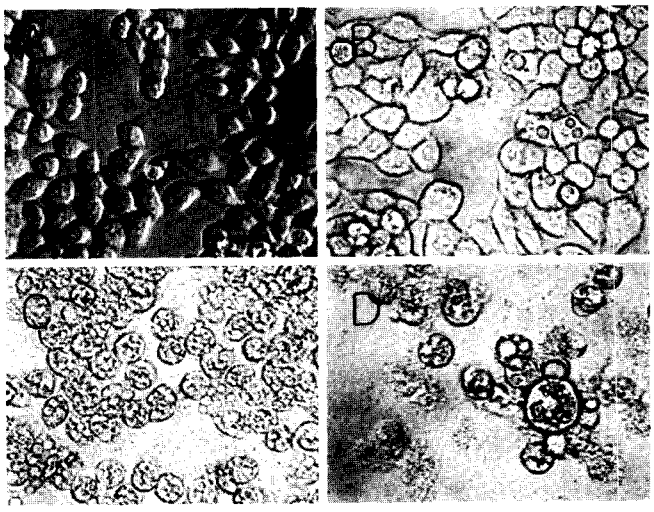

Fig. 6. Microscopic assessment of vacuolar degeneration in HeLa cells.
Pictures were taken by magnifying 200-fold the cells grown for 2 days with 10 mM NH_4Cl and the following additives: A, none; B, 2 mM DEPC; C, VacA and 2 mM DEPC; D, *H. pylori* cells. Note that *H. pylori* colonies are formed around the spherically degenerated cells (D).

by pinocytosis [20], which involves VacA reorganization enriched for late endosomal or lysosomal markers. In fact, recent data suggest that the apparent increase in the sensitivity of cells to VacA may result from the change of expression of one of the membrane-associated Rho family [15] or due to presence of some nonspecific membrane-mobilizing agents, such as 12-O-tetradecanoylphorbol-13-acetate (TPA) [7] or Na-butyrate. Nevertheless, the pathogenic mechanism associated with vacuolar degeneration by *H. pylori* VacA still remains unclear.

In this study, we found that although VacA itself was strongly inhibited by DEPC, DEPC allowed the toxin to cause not only vacuolar degeneration but also growth retardation and rate increase of VacA uptake. Based on these observations, together with the fact that despite of its apparent inhibitory effect on intracellular peptidase action, DEPC did not perturb PSP hydrolysis inside the cells, we conclude that this agent modifies only the plasma membrane, so that VacA protein is able to penetrate easily. Similar phenomena were also observed with intact *H. pylori* or its culture filtrate, suggesting that *H. pylori* would secrete some factor to make the cells sensitive to VacA attack. In the literature, *H. pylori* culture filtrate was reported to affect cellular functions such as cell migration, proliferation, maturation of procathepsin D, or epidermal growth factor degradation [26, 28].

H. pylori is basically noninvasive, because of the following aspects: i) Intercellular spaces just beneath the mucosal layer have long been identified as niches for bacterial colonization [13]: ii) The prevalence of colonizing human stomach antrum highly exceeds the acidic fundus [3]: iii) Histological studies also suggest that *H. pylori* is able to adhere to the apical area of epithelial cells without any apparent derangement of the mucus layer [1, 14]; and iv) The majority of *H. pylori*-infected persons remain asymptomatic. These considerations make it clear that *H. pylori* can colonize by circumventing acid, regardless of the clinical outcome. Thus, our trial to assess the extent of vacuolation by VacA is indicative of whether or not host cells surrounding *H. pylori* are fatally degenerative. The present results suggest a possible disruption of the mucosal acid-independent repair system, which is of great importance in establishing the pathogenesis of *H. pylori*. In addition, it is suggested that fatal degeneration in the course of vacuolation by VacA is a natural but random process that is under the stringent control of a rate-limiting kinetics at the level of initial VacA transport. This, in turn, suggests a possible pathogenesis by inflammation-independent apoptosis [21] in which host cells under vacuolation contiguous to actively growing bacteria are believed to be fatal.

In summary, we have shown a correlation between vacuolation rate and cell degeneration, and suggest an acid-independent mechanism for the pathogenesis of *H. pylori*, resulting from an event that involves a transition state such as vacuolar degeneration. Moreover, the process is thought to be closely dependent upon the rate of VacA transport. In this respect, we hypothesize that increasing the VacA susceptibility of the plasma membrane would increase the probability of pathogenesis associated with *H. pylori* infection. Finally, the putative factor to mobilize plasma membrane should be investigated further.

Acknowledgments

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