Association between *Helicobacter pylori* VacA antigens and gastric cancer depends on the detection method used: immunoblot versus neutralization of the vacuolating activity of VacA

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There are inconsistent findings on the association between *Helicobacter pylori* anti-VacA antibodies and gastric cancer (GC) risk. The lack of optimally sensitive and specific methods to detect anti-VacA antibodies may partly be responsible for this discrepancy. The aim of this study was to compare the association between GC and the presence of anti-VacA antibodies using two different detection methods. A secondary analysis of sera collected in a hospital-based case-control study in three geographical areas of Mexico was performed. Anti-VacA antibodies were determined by a neutralization assay and an immunoblot assay in serum samples of 203 histologically confirmed GC cases and 430 age- and sex-matched controls. *H. pylori* IgG status was determined by immunoblotting and *H. pylori* CagA status data was available for this study. Unconditional logistic regression models were used to estimate the association between anti-VacA antibodies and GC by histological type (diffuse and intestinal). Anti-VacA seroprevalence was higher using neutralization compared with immunoblotting: 68.5 vs 44.3 % for cases and 60.5 vs 44.2 % for controls. A significant association between anti-VacA antibodies and diffuse GC was found using neutralization [odds ratio (OR)=1.69, 95 % CI 1.08–2.66], but the association did not remain significant after adjusting for CagA status (OR 1.37, 95 % CI 0.81–2.32). No association between anti-VacA antibodies and GC was found when using immunoblotting. Thus, the association between anti-VacA antibodies and GC partly depends on the detection method used. These results do not support an independent role for VacA in GC risk in the presence of CagA seropositivity and strengthen the importance of CagA as a potential risk factor for GC.

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INTRODUCTION

Less than 3 % of subjects infected with *Helicobacter pylori* develop gastric cancer (GC) (Peek & Blaser, 2002). Several virulence factors produced by *H. pylori* are now thought to be implicated in the carcinogenesis process (Xia & Talley, 2001; Hocker & Hohenberger, 2003). Of the bacterial factors associated with GC, the most extensively studied are probably the cytotoxin CagA (Hatakeyama, 2003) and the vacuolating cytotoxin VacA (de Figueiredo Soares *et al.*, 1998). However, other virulence factors also have activities that could induce cell transformation by affecting cell metabolism and/or causing tissue damage in general. Among these are the BabA2 adhesin, whose presence is associated with the existence of pre-neoplastic gastric lesions (Yu *et al.*, 2002), and a urease, an enzyme that

releases ammonia, which has been suggested to be the cause of an acceleration of cytokine-induced apoptosis in gastric epithelial cells (Igarashi *et al.*, 2001). A novel apoptosis-inducing protein encoded by the *H. pylori* HP1118 gene has been isolated (Shibayama *et al.*, 2003), and recently it was demonstrated that the co-chaperonin GroES is implicated in the induction of pro-inflammatory cytokine and neoplastic changes (Lin *et al.*, 2006).

The *H. pylori* VacA cytotoxin is a virulence factor potentially linked to GC. VacA causes cell vacuolation in cell culture, produces significant epithelial cell damage and increases the level of apoptosis in epithelial cells (Galmiche *et al.*, 2000; Kuck *et al.*, 2001; Cover *et al.*, 2003); this in turn enhances the hyperproliferative response, altering the balance of gastric homeostasis, which changes gastric epithelial cell turnover and permits the persistence of mutated cells. Studies suggest that these processes may promote carcinogenesis (Galmiche *et al.*, 2000; Xia & Talley, 2001).

Abbreviations: GC, gastric cancer; OR, odds ratio; SVA, supernatants with vacuolating activity.

The clinical and epidemiological evidence regarding the relationship between VacA and GC is unclear (de Figueiredo Soares *et al.*, 1998; Shimoyama *et al.*, 1999; Yamaoka *et al.*, 1999; Enroth *et al.*, 2000; Figueroa *et al.*, 2002). Among patients with GC, some clinical studies have reported positive correlations between antibody host response to VacA and the presence of VacA-positive *H. pylori* strains (de Figueiredo Soares *et al.*, 1998; Garza-Gonzalez *et al.*, 2004). Some epidemiological studies have shown an increased risk of GC in seropositive VacA subjects (Enroth *et al.*, 2000; Figueroa *et al.*, 2002), but not all studies have reported these findings (Shimoyama *et al.*, 1999; Yamaoka *et al.*, 1999). Two possible explanations for the inconsistent reporting of the VacA/GC association are: (i) the lack of control for key confounding factors such as *H. pylori* CagA status, and (ii) significant differences in the sensitivity and specificity of the immunoblot kits used to determine the *H. pylori* VacA status; among these are: Helicoblot (HB2.0 or HB2.1; Genelabs Diagnostics) (de Figueiredo Soares *et al.*, 1998; Enroth *et al.*, 2000; Garza-Gonzalez *et al.*, 2004), the Chiron RIBA Recombinant Immunoblot assay (Chiron) (Shimoyama *et al.*, 1999; Yamaoka *et al.*, 1999) and other homemade immunoblot assays (Hirai *et al.*, 1994; Rudi *et al.*, 1997; Figueroa *et al.*, 2002).

Here, we compared the association between GC and the presence of anti-VacA antibodies using two different detection methods; in addition, we adjusted for CagA status. The study was conducted with serum samples from Mexico, where approximately 80 % of the adult population are seropositive for *H. pylori* (Torres *et al.*, 1998) and where GC is still a leading cause of death by cancer (Secretaría de Salud, 1993). Unlike the rest of the world (Parkin *et al.*, 1997), the incidence of this type of cancer is not decreasing.

METHODS

This study undertook a secondary analysis of serum samples obtained between 1994 and 1996 in a hospital-based case-control study that was conducted in three distinct geographical areas of Mexico. The areas were selected based on the variability of *H. pylori* seroprevalence and dietary patterns. More detailed information about the methodology of this study has been published elsewhere (López-Carrillo *et al.*, 2003).

Cases. Inclusion criteria for case subjects were: histologically confirmed adenocarcinoma of the stomach, either intestinal or diffuse type; no previous history of any other cancer; at least 20 years of age; and resident in the study area for at least the previous 6 months. Eligible cases were recruited from 13 tertiary, public, social security hospitals in Mexico, distributed as follows: seven from Mexico City (Federal District), three from the city of Puebla (State of Puebla) and three from the city of Merida (State of Yucatan). A single expert cancer pathologist determined the histological variety of the tumour (intestinal or diffuse), following the criteria established by Lauren (1965). Information about the anatomical subsite location of the tumour was not available for this study. In total, 281 patients who met all of the eligibility criteria were identified (approx. 75 % of the total number of GC patients reported to the Mexican National Cancer Registry during the study period of recruitment for the study areas)

and 261 agreed to enrol in the study, giving a participation rate of 92.9 %.

Controls. For each case subject, we recruited a minimum of two age-, sex- and residence-matched control subjects. Controls were randomly identified in selected departments of the participating hospitals. Eligibility criteria included: no previous diagnosis of malignant tumour, diet-related disease (gastritis, peptic ulcer, liver cirrhosis or diabetes mellitus) or immunosuppressive condition; same age (± 5 years) as the index case; and resident in the same study area as the index case for at least the previous 6 months. The most common diagnoses among controls were: circulatory system diseases (other than hypertension) (18.8 %); diseases of the nervous system and the sensory organs (except psychiatric disorders) (16.1 %); osteomuscular and connective tissue disorders (14.4 %); injuries and poisonings (10.9 %); and respiratory diseases (10.0 %). Other less common diagnoses were genitourinary diseases and skin problems. We also recruited control subjects who were healthy companions of patients and those attending the hospitals for preventive care purposes, such as vaccination. The participation rate for the control group was 94.6 % (523 out of 553 eligible subjects).

Interviews. After informed consent had been obtained from each subject, they were questioned about their socioeconomic, clinical and dietary characteristics. The interviews were performed in the participating hospitals by trained personnel.

Serum samples. Blood samples (10 ml) were drawn from each subject. After centrifugation, serum aliquots were obtained and stored at -70°C until the samples were tested. Serum samples from a total of 203 cases and 430 controls were available for this study.

The *H. pylori* status of each subject was determined. *H. pylori* IgG antibodies were determined by immunoblot as described below. The *H. pylori* CagA status was available for this study and the ELISA procedures used to determine it have been described elsewhere (López-Carrillo *et al.*, 2004).

Production of cytotoxin and assessment of vacuolating activity. A high level toxin-producing *H. pylori* strain (7494, s1/ml genotype and *cagA*⁺) was used as the source for vacuolating cytotoxin preparation. Colonies obtained from one blood agar plate [tryptic soy agar (Difco) supplemented with 5 % sheep blood plus $10\ \mu\text{g}$ vancomycin ml^{-1}] were inoculated into 100 ml sulfite-free *Brucella* broth containing 0.2 % cyclodextrin (Sigma) and cultured as described previously (Ayala *et al.*, 2006). The cultures were centrifuged at $15\ 000\ g$ for 20 min at 4°C . The supernatants were then pooled and this fraction was termed 'supernatants with vacuolating activity' (SVA). Analysis by 10 % SDS-PAGE showed that this fraction was composed of proteins of different molecular mass, among them a band corresponding to VacA ($\sim 87\ \text{kDa}$). The SVA fraction was used as the source of VacA for the neutralization assays, as well as antigen for immunoblot analysis. Determination of vacuolating activity for the SVA fraction was conducted essentially as described by Cover *et al.* (1990). The assay was carried out by the addition of a fixed amount of total SVA protein ($6.1\ \mu\text{g}$) to confluent HeLa cells. To estimate quantitatively the vacuolating activity of the SVA fraction, a neutral red uptake assay was used as reported elsewhere (de Bernard *et al.*, 1998) and is described below. The protein concentration of the SVA was determined using the Lowry method (Lowry *et al.*, 1951).

H. pylori anti-VacA antibody status was determined by two methods: a VacA-neutralizing antibody assay and an in-house immunoblot assay.

Neutralization of VacA cytotoxin activity. The serum from each patient was inactivated as described elsewhere (Cover *et al.*, 1992) and

then diluted with Dulbecco's modified Eagle's medium (DMEM) cell-culture medium. Inhibition of VacA activity using patient sera was performed according to a method reported elsewhere (Cover *et al.*, 1992) and modified as follows: equal volumes of *H. pylori* SVA containing 6.1 µg total protein and serum from each subject were pre-incubated for 1 h at 37 °C. The starting amount of protein in each serum sample was 9.5 µg and ten further twofold dilutions from 1:4 to 1:2048, were carried out in DMEM culture medium in 96-well plates. Next, 100 µl from each well containing the SVA/serum mixture was transferred to a second 96-well plate containing confluent HeLa cells (7×10^3 cells per well). The plates were incubated for 18 h at 37 °C with 5 % CO₂. For each serum sample, two controls were included: (i) HeLa cells with SVA but without serum (positive control), and (ii) HeLa cells with DMEM culture medium alone (negative control). Experiments were carried out in duplicate. Both vacuolization and inhibition were evaluated by neutral red uptake assay (de Bernard *et al.*, 1998). Cells were stained for 8 min with 0.05 % neutral red. After washing the cells twice with 0.3 % BSA in PBS, intravacuolar neutral red was extracted with 70 % ethanol and 0.37 % HCl. A₅₄₀ was determined using a Multiskan EX ELISA reader (Thermo Electron). All assays were performed in duplicate. Results were expressed as the mean A₅₄₀ of experimental wells, minus background A₅₄₀ (the mean A₅₄₀ of wells containing HeLa cells incubated with medium only). The net A₅₄₀ value was used to calculate the cut-off value.

The cut-off point was determined by comparing the net A₅₄₀ value of wells containing both the SVA fraction and the serum being tested. The net A₅₄₀ values obtained for each of ten serial dilutions (described above) were compared by analysis of variance. The positive/negative cut-off point was determined using the lowest dilution for which a statistically significant difference was observed in the mean net A₅₄₀ between two consecutive dilutions. The cut-off point was set as 0.16 absorbance units at a dilution of 1:32. Thus, a serum was judged to be positive (i.e. showed neutralizing activity) if the A₅₄₀ value was lower than 0.16 at a dilution of 1:32. Sera with A₅₄₀ ≥ 0.16 were considered negative (i.e. no neutralizing activity).

***H. pylori* and VacA cytotoxin immunoblot assay.** The SVA fraction was concentrated approximately 50-fold by ultrafiltration with a 10 kDa cut-off (Millipore). Proteins were separated by 10 % SDS-PAGE and transferred to nitrocellulose membranes (Millipore). The filter was blocked for 1 h with buffer containing 10 % (v/v) skimmed milk in 20 mM Tris/HCl (pH 7.5) containing 0.5 M NaCl. The membranes were cut into strips and incubated overnight at room temperature with serum samples from each patient at a 1:200 dilution. The strips were washed and further incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-human IgG (Sigma). A serum sample was considered to be *H. pylori*-positive if three or more immunoreactive bands were present on the strip; otherwise, the sample was considered negative. The sample was also judged to be VacA-positive if a band with a molecular mass of ~87 kDa was recognized. To confirm that the 87 kDa band corresponded to VacA, one strip was incubated with anti-VacA antibody (bN-20; Santa Cruz Biotechnology). Colour reactions were developed with 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium.

Statistical analyses. The general characteristics of cases and controls (age, sex, etc.), as well as *H. pylori* status (IgG, CagA and anti-VacA antibodies), were compared using Student's *t* test and χ^2 statistics. Crude and adjusted risks (by age and CagA status) for GC according to the presence of *H. pylori* IgG antibodies and VacA status were estimated using unconditional logistic regression models for all adenocarcinomas and then stratified for histological type of GC (i.e. intestinal and diffuse).

RESULTS AND DISCUSSION

The general characteristics of the study population are presented in Table 1. Age, sex and residence distribution were similar, and no significant difference was found between cases and controls regarding socioeconomic level. Intestinal GC was more frequent than diffuse GC in this population.

H. pylori status and anti-VacA and anti-CagA seroprevalence

There was a significantly higher proportion of cases positive for *H. pylori* IgG compared with the controls (91.1 vs 83.2%; Table 2). A statistically significant difference in CagA-positive status was also found between cases and controls (78.6 vs 66.0 %).

In this study, VacA seroprevalence was analysed by two methods: neutralization of vacuolating activity of VacA cytotoxin *in vitro* and immunoblot assay. VacA seroprevalence was higher by neutralization, compared with immunoblotting: 68.5 vs 44.3 % for cases and 60.5 vs 44.2 % for controls (Table 2). A borderline significant difference was found between cases and controls for the presence of anti-VacA antibodies (68.5 vs 60.5 %) when these were determined by neutralization. In contrast, when immunoblotting was used to detect anti-VacA antibodies, lower and similar percentages were found in both cases and controls (44.3 and 44.2 %, respectively). The discrepancy between the two methods may be due to the fact that most

Table 1. General characteristics of the study population

Characteristic	Cases (n=203) (%)	Controls (n=430) (%)	P value*
Age (years)			0.883
<40	10.8	10.0	
40–50	18.7	18.1	
50–60	21.2	24.2	
60–70	29.1	30.0	
>70	20.2	17.7	
Sex			0.803
Male	43.8	42.8	
Female	56.2	57.2	
Residence			0.328
Mexico City	30.5	28.6	
Puebla State	43.9	40.0	
Yucatan State	25.6	31.4	
Change in socioeconomic level†			0.560
Same or less	50.5	48.0	
Improvement	49.5	52.0	
Histological type			
Intestinal	37.2	–	–
Diffuse	62.8	–	–

*P value from χ^2 test.

†Current level compared with socioeconomic level in childhood.

Table 2. *H. pylori* status (including virulence factors) in the study population

Positive for:	Cases (n=203) (%)	Controls (n=430) (%)	P value*
IgG (Western blot)	91.1	83.2	0.008
VacA (immunoblot)	44.3	44.2	0.984
VacA (neutralization assay)	68.5	60.5	0.051
CagA (ELISA)	78.6	66.0	0.002

*P value from χ^2 test.

anti-VacA antibodies present in *H. pylori*-infected patients recognize conformational epitopes of VacA, whilst linear epitopes of VacA are recognized to a lesser degree (Figueiredo *et al.*, 2001). This weak reactivity of VacA in immunoblots using sera from patients with active *H. pylori* infection has been reported previously (Lepper *et al.*, 2004). The failure to recognize linear VacA epitopes could potentially result in differences in sensitivity and specificity between immunoblotting and neutralization. Therefore, detection of anti-VacA antibodies by inhibition of VacA activity *in vitro* should produce more reliable data than immunoblot assays. It is important to note that human serum from *H. pylori*-infected patients, as well as from uninfected persons, contains non-specific VacA-neutralizing activity that does not depend on anti-VacA IgG (Cover *et al.*, 1992). However, such an effect would not have affected our results at the dilution used (1:32), as the amount of serum protein at the positive/negative cut-off point was very low (~0.30 µg), so any non-specific neutralizing activity would be negligible.

Association of virulence factor with GC

Table 3 shows that although immunoblotting and neutralization both detected anti-VacA antibodies, only when the neutralization assay was used were we able to find a significant odds ratio (OR 1.69, 95 % CI 1.08–2.66). A

significant association between *H. pylori* IgG antigens and GC risk was also detected (OR 2.37, 95 % CI 1.15–4.89) (data not shown).

It is well accepted that individuals infected with *H. pylori* strains expressing both CagA and VacA are at higher risk of developing GC (Rudi *et al.*, 1997; Enroth *et al.*, 2000; Figueroa *et al.*, 2002). However, there is still little information to show the extent to which the presence of both virulence factors contributes to the development of GC. Studies of VacA biological activity (Galmiche *et al.*, 2000; Kuck *et al.*, 2001; Cover *et al.*, 2003; Karita *et al.*, 2005) suggest that it plays an indirect role in the development of GC, mainly by inducing apoptosis, which results in the atrophy associated with the carcinogenic process. Although our results showed a significant association between anti-VacA antibodies and diffuse GC when detected by neutralizing antibodies, the association did not remain significant after adjusting for anti-CagA antibodies (OR 1.37, 95 % CI 0.81–2.32). Thus, our results strengthen the importance of CagA as a potential risk factor for GC, despite the fact that its presence improves the assessment of GC risk for VacA.

VacA and CagA are involved in different steps of the pathogenic process. VacA plays an essential role in the initial colonization of *H. pylori* and the subsequent epithelial cell damage of the stomach mucosa (Salama *et al.*, 2001). VacA also suppresses the local immune response, which promotes the presence of a chronic, persistent *H. pylori* infection (Gebert *et al.*, 2004). CagA-positive *H. pylori* strains possess an intact Cag pathogenicity island, which is associated with activation of the inflammatory response and mucosal immunity, as well as with altered regulation of multiple genes associated with proliferation and differentiation of gastric epithelial cells. The function of the CagA protein itself is not yet clear, but recent reports have associated it with transformation of the gastric epithelium. This might indicate that the development of GC would only occur if the *H. pylori* infection is chronic and if the infecting strain is CagA-positive (Amieva *et al.*, 2003; Hatakeyama, 2004). Chronic inflammatory

Table 3. ORs for the effect of *H. pylori* VacA on the risk of intestinal and diffuse GC

VacA status	Intestinal GC			Diffuse GC		
	Cases/controls (n)*	OR (95 % CI)†	OR (95 % CI)‡	Cases/controls (n)*	OR (95 % CI)†	OR (95 % CI)‡
Immunoblot						
Negative	36/239	1.0	1.0	69/239	1.0	1.0
Positive	32/191	1.11 (0.66–1.86)	0.89 (0.52–1.55)	46/191	0.83 (0.55–1.27)	0.68 (0.43–1.06)
Neutralization assay						
Negative	27/170	1.0	1.0	32/170	1.0	1.0
Positive	41/260	0.99 (0.59–1.67)	0.71 (0.39–1.30)	83/260	1.69 (1.08–2.66)	1.37 (0.81–2.32)

*Cell numbers that do not add up to the total sample size are due to missing values.

†Crude OR.

‡OR adjusted by age and CagA status.

changes are linked to cancer, thus explaining the relative importance of CagA versus VacA in GC. However, we cannot exclude the participation of the co-chaperonin GroES of *H. pylori* in the induction of inflammation and neoplastic changes (Lin *et al.*, 2006), reflecting the multiple pathogenic possibilities displayed by this bacterium.

VacA is a highly expressed protein *in vivo* (Boonjakuakul *et al.*, 2004) and some studies have shown that carriers of *H. pylori* strains that efficiently produce VacA are at higher risk of GC. However, few studies have evaluated the relationship of GC with the presence of anti-VacA antibodies (Rudi *et al.*, 1997; de Figueiredo Soares *et al.*, 1998; Enroth *et al.*, 2000; Figueroa *et al.*, 2002) and results have been inconsistent (Shimoyama *et al.*, 1999; Yamaoka *et al.*, 1999). Thus, the anti-VacA/GC association is questionable, and earlier controversial results may partially be explained by their different methods and the criteria of the assay.

This study showed that the association between anti-VacA antibodies and GC risk depends not only on the type of serological test used to detect anti-VacA antibodies and CagA, but also on the CagA status. This last situation does not support an independent role for VacA in GC risk.

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