High prevalence of cytotoxin positive *Helicobacter pylori* in patients unrelated to the presence of peptic ulcers in Japan

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Abstract

Background—It has been reported that infection with vacuolating cytotoxin positive *Helicobacter pylori* strains is associated with gastroduodenal disease in Western countries.

Aims—To evaluate the prevalence of cytotoxin producing strains among patients with *H. pylori* infection in relation to gastrointestinal diseases in Japan.

Patients—Ninety seven patients undergoing endoscopy.

Methods—A Western blot assay was conducted to detect serum antibodies against the cytotoxin using recombinant cytotoxin (VacA protein) as an antigen. To obtain a purified recombinant cytotoxin, the *vacA* gene (2233 nucleotides) was cloned into an expression vector to produce the protein (744 amino acids), which was expressed in *Escherichia coli*.

Results—Serum IgG antibodies to the cytotoxin were present in 85%, 95%, 95%, and 100% of infected patients with gastric ulcer (n=26), duodenal ulcer (n=21), chronic gastritis (n=19), and endoscopically normal mucosa (n=14), respectively.

Conclusion—The western blot method using recombinant VacA protein is simple and useful for detecting antibody to vacuolating cytotoxin. This method showed antibodies against cytotoxin were highly prevalent, even in subjects with endoscopically normal mucosa in Japan, indicating that the cytotoxin may not be an independent cause of gastrointestinal diseases induced by *H. pylori* infection.

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Keywords: stomach; *Helicobacter pylori*; *vacA*; ulcer; gastritis

Helicobacter pylori is a Gram negative bacterium which resides in the human stomach. The prevalence rate of its infection varies between countries, and a relatively high prevalence has been reported in Japan compared with other developed countries.^{1,2} Approximately 70% of the Japanese population aged over 40 is reported to be infected with the pathogen.

The infection is usually well tolerated with few symptoms, if any, in the majority of patients; nevertheless, it may play a significant role in the pathogenesis of peptic ulceration. This is highlighted by the reduction in the pep-

tic ulcer relapse rate after successful eradication of this pathogen by antibiotics.^{3,4} However, the pathophysiological mechanisms for the different clinical diseases induced by *H. pylori* infection are not well understood. Several host factors, such as smoking,⁵⁻⁸ male sex,⁹ and blood type O,¹⁰ have been reported to predispose the host to the development of peptic ulceration.

Recent studies have suggested that differences in the virulence of *H. pylori* strains may influence the wide spectrum of gastrointestinal diseases. One of the claimed pathogenic bacterial factors is the 139 kDa vacuolating cytotoxin,^{11,12} known as the *vacA* gene product. This cytotoxin has been reported to be produced by approximately 50% of *H. pylori* strains isolated in Western countries, and has been found to be associated with peptic ulceration.¹³⁻¹⁸ Tee *et al*¹⁸ reported that the highest percentages of the strains producing cytotoxin were found in subjects with peptic ulcer disease (gastric ulcer, 65% of 20 patients; duodenal ulcer, 66% of 73 patients) compared with only 38% of those with non-ulcer dyspepsia (63 patients).

Antibodies in human sera against the vacuolating cytotoxin have often been assessed by using the neutralising assay.^{16,19} In Japan, Hirai *et al*²⁰ reported a high prevalence of antibody (100%) in sera of six patients with gastric carcinoma, using the antibody mediated neutralising assay against the cytotoxin, suggesting that there is a close relation between infection with cytotoxin producing strains and gastric cancer. The methodology of the neutralising assay, however, is too complicated for large scale analysis.

In order to assess the serum antivacuolating cytotoxin antibody which is thought to indicate infection with cytotoxin positive *H. pylori* strains, we have cloned the *vacA* gene, purified the recombinant cytotoxin protein expressed in *Escherichia coli*, and conducted a western blot assay to detect the antibody against the toxin in patients with and without various gastrointestinal diseases.

Methods

BACTERIAL STRAINS AND CULTURE CONDITIONS

H. pylori strain ATCC (American Type Culture Collection) 43526, which is positive for vacuolating cytotoxin, was used to clone the *vacA* gene. *H. pylori* strain Tx30a, vacuolating cytotoxin negative strain (Tox-),²¹ was kindly

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Figure 1: Schematic representation of plasmid constructs used for sequence analysis of the *vacA* gene and expression of cytotoxin protein in *E. coli*. P, promoter; 6x His, histidine hexamer tag; LP, leader peptide; OM exp, outer membrane exporter.

provided by Dr JC Atherton (Vanderbilt University, Nashville, Tennessee, USA). *H. pylori* was cultured in Brucella broth culture medium (Becton Dickinson, Cockeysville, Maryland, USA) containing 10% fetal bovine serum (Cansera International Inc., Canada) in a microaerophilic atmosphere generated by CampyPak-Plus (Becton Dickinson) at 37°C for 48 hours. *E. coli* JM109 was used for the cloning experiments. Recombinant bacteria were grown in Luria-Bertani broth or agar plates containing ampicillin.

CLONING OF *vacA* GENE

H. pylori ATCC 43526 cells were cultured, harvested, and lysed in TNE (20 mM Tris-HCl (pH 7.5)/0.1 M NaCl/1 mM EDTA) buffer with 0.3% SDS and 0.2 mg/ml of proteinase K at 60°C overnight. The DNA was purified by phenol extraction, chloroform extraction, and precipitation with ethanol. Oligonucleotide primers with a PstI restriction site at the 5' terminus were designed to amplify the full length of the *vacA* gene open reading frame (ORF; 3864 bp)²² and used for polymerase chain reaction (PCR) amplification. Primer sequences were as follows: sense: 5'-AGCTGCAGCATGGAAATACAACAAACACACCGCA-3' and antisense: 5'-AGCTGCAGCTTAGAAACTATACCTCATTCTTA-AA-3'. PCR products (3891 bp), which contain the complete *vacA* gene ORF, were digested with PstI and cloned into the PstI site of pBluescript II SK (+) (Stratagene, La Jolla, California, USA), resulting in pBS*vacA* (fig 1). Nucleotide sequences of the insert were determined by the dideoxy chain termination procedure.²³

EXPRESSION AND PURIFICATION OF RECOMBINANT *VAC* PROTEIN

The pBS*vacA* was digested with PstI and KpnI, and the *vacA* fragment encoding amino acids 1 to 744 was cloned in frame with the sequence coding for the histidine tag in the PstI-KpnI sites of pQE32 (Qiagen, Chatsworth, California, USA), resulting in pQE32*vacA* (fig 1). *E. coli* strain JM109 was transformed by pQE32*vacA* and recombinant protein was purified on Ni-nitrilotriacetic acid resin (Qiagen) as described by the manufacturer. Briefly, the recombinant protein was bound to the column in 6 M guanidine hydrochloride/0.1 M sodium phosphate/0.01 M Tris (pH 8.0) and eluted with 8 M urea/0.1 M sodium phosphate/0.01 M Tris (pH 5.9).

IMMUNISATION OF RABBITS

Antibodies were raised in rabbits against the recombinant VacA protein and a synthetic oli-

gopeptide, VA-1. The VA-1 peptide, TSYKD-SADRTTR (*vacA* residues 176–187 of *H. pylori* strain ATCC 43526), designed to have antigenicity, was synthesised according to the multiple antigen peptide (MAP) method²⁴ using the Shimadzu PSSM8 peptide synthesiser as described by the manufacturer. The amino acid sequences comprising the VA-1 peptide are conserved in Tox+ strains. In Tx30a, a Tox- strain, *vacA* sequences (TSFKDGANRTTR) differ from the VA-1 peptide by three amino acids. The VA-1 peptide was used to obtain the antivacuolating cytotoxin antibody. New Zealand White rabbits, weighing approximately 2.5 kg, were immunised by intradermal injections of 25 mg purified recombinant VacA protein or 300 mg VA-1. The rabbits were injected with a 1:1 emulsion of the antigen in complete Freund's adjuvant. Subsequently, three booster immunisations were performed every two weeks as a 1:1 emulsion in incomplete Freund's adjuvant. The antisera were collected two weeks after the last booster.

DETECTION OF *VAC* EXPRESSION OF *H. PYLORI* ISOLATES BY WESTERN BLOT ASSAY

H. pylori culture supernatant was run on 10% SDS-polyacrylamide gels, and the proteins were transferred to nitrocellulose membranes by electroblotting. The membranes were then probed with antirecombinant *vacA* or anti-VA-1 rabbit antisera and ¹²⁵I-goat antirabbit IgG.

SOURCES OF HUMAN SERUM

Serum samples were obtained from 97 patients (64 men, 33 women; mean age 51.7 years) who underwent gastroduodenal endoscopy and were tested for *H. pylori* infection by culture, rapid urease test, and the PCR amplification of the urease gene (*ureA*) in gastric juice.²⁵ No patient was taking non-steroidal anti-inflammatory drugs. The sera were kept at -20°C pending analysis.

DETECTION OF SERUM IgG ANTIBODIES TO *H. PYLORI*

Serum samples were tested for the presence of antibodies against *H. pylori* by enzyme linked immunosorbent assay (ELISA), using a commercial kit, Helico G ELISA Kit (Porton Cambridge, Newmarket, UK), which detects the human anti-*H. pylori* IgG against total cell lysates of *H. pylori*.

WESTERN BLOT FOR DETECTION OF SERUM ANTIBODIES TO *VAC*

After separation by 10% SDS-PAGE, the recombinant *vacA* protein was transferred to a nitrocellulose membrane by electroblotting. The membrane strips were blocked with 3% skimmed milk overnight, incubated with a 1/100 dilution of the patient's serum, and shaken constantly at room temperature for two hours. After washing, protein bands were detected using the Vectastain ABC kit (Vector Laboratories Inc.), according to the manufacturer's instructions.

43526	1:MEIQTHKRIKPLVSLVGLV-S-I---TPQGS-H---A-AFFTIVIPAIVGGIATGAAGVTSGLLSMLGKQAEANKTPKPKVVRITQAGRGFN	91
60190	1:.....N.....T.....G.....K.....	91
Tx.30a	1:.....II.....V.MGTELGA.....NDPTI.SESR.....Q.....D	100
43526	92:NFPKKEYLYKSLSSKIDGNDGNGAARHYVYGGGQNNKLEVDKDAVGYKLSGLNFTGGDLOVMKATRLRGQFGNGSFTSYKSDADRTTRVDFN	191
60190	92:.....N.....R.....	191
Tx.30a	101:.....Q.....D.....Q.....N.....F.....G.....M.....D	200
43526	192:AKNILDINFLEINNVGSGAGKASSTVLTQASEGITSSKNAEISLYGATLML--AS-SSVKLMGAVYNGRLQYVGYLAPSYSTINTSKVTGEVNFN	288
60190	192:.....N.....N.....	288
Tx.30a	201:.....V.....K.....RE.....VSS.MQ.....D.....Y.....K.....D.....Q.....M.....R	300
43526	289:HLTVGDHAAQAGITASNKTHIGTDLNQSAGLHIAIPEGGYKPKPKPSHTTQNNAMNQNSAQNNNTQVNPMSAQKTEIQPTQVINGPFGGG	388
60190	289:.....SG.K.DK.E.S.....S.....T.....V.....D.....A.....	385
Tx.30a	301:.....A.....Q.....NE.....N.....S.....T.....E.....K.....D.....N.....P.....K.....D.....D.....A.....	377
43526	389:KDTVYVNIENRINTNADGTIRVGGKASLTNAHMEKGGKINLSQASGSLLENLTGHTVDPGLRVYVYVGGVALAGSNAHFEKAGTDTKNGTATF	488
60190	386:.....D.....K.....K.....F.....M.....V.....T.....S.....V.....V.....	485
Tx.30a	378:.....FHL.....K.....LEA.....F.....S.....E.....V.....T.....E.....T.....A.....I.....E.....N.....A.....	477
43526	489:NNDISLGR-----F-GN--L-K-----VDAHTANFKGID-T-GNGGF--TTLDFCGVTOKVIINKLITASTNAIKNSHINDLVTNNGVSG	563
60190	486:.....V.....Q.....F.....S.....N.....N.....N.....V.....F.....E.....T.....K.....	560
Tx.30a	478:.....H.....KAVNLAVDAHTAN.....N.....Y.....G.....STNLR.NG.....H.....EN.....A.....KSDN.LNTS.....S.....K.....N.....T.....A.....N.....FD.KE.V.....RVQ.F.	577
43526	564:EYTHFSEDIGSGSRINTVLETRTSIFSGGVKFSGEKLVIDEFYSPMYLDARKIKHVEITRKFSSTPENPWTGSKLNNLTLGQNAAMDYSQFS	663
60190	561:.....F.....	660
Tx.30a	578:Q.....I.....G.....N.....OF.....GV.S.Q.....YSPAY.....T.....G.....K.....I.....HA.....F.....VTD.....K.....RELFGA.G.TA.KTG.....NS.....S.....QNDL	677
43526	664:NLTIQDGFIMQGTINYLVRGGKATLNYGMAAAMFNNIDISATGFYKPLIKMSAQDLIKTEHYLLKAKIIGYGVST-G-T-NGI--SMVNLQEF	758
60190	661:.....	755
Tx.30a	678:D.....H.....T.....M.....LF.QD.R.....A.....HQ.S.I.....LV.....T.....N.....N.....T.....K.....V.....RN.D.ML.GVQ.ASYDN.SA.....T.....Q.....	777
43526	759:KGRALYNNRNRDTCVVR--N-DOIKACGAGTNGSMVTTTPNKKYLTGAKMKNGISKTANGSKISVYLYGNSPTENGNTNLPMTTNSARSANY	856
60190	756:.....E.....	853
Tx.30a	778:.....E.....KDNLN.....NN.E.....D.....T.....N.....NTT.A.MGN.....NSTTD.....N.....F.....S.....	877
43526	857:ALVKAPFAHSAFNLVAZINQHECGTIESVCLAMISKIDITLYTHSGAKGRILLQTLIDSHDAGYVRQMDNTSTGETKQLNAATTLNIAISLEHK	956
60190	854:.....I.....DF.....F.....S.....AN.....Q.....A.....T.....A.....AN.....T.....	953
Tx.30a	878:.....I.....DF.....F.....S.....AN.....Q.....A.....T.....A.....AN.....Q.....	977
43526	957:TSSLQTLSLSMAMINSLRVLSRKHTNNIDFSAKRLQALQKQFASLESAAEVLVQFAPKYKPTNMVNAATGGASLMGSSNASLYGTSAGVDAYLNGQ	1056
60190	954:.....R.....	1053
Tx.30a	978:.....G.....H.....	1077
43526	1057:VEATVGGFGSYGSSFSNRSANLSGNTNTHGGVYSIFANQHEFDFEAQGLGSDQSSLNFKSALLQDLNQSYMYLAYSATRASGYDFAFKMLVL	1156
60190	1054:.....N.....Q.....A.....F.....R.....	1153
Tx.30a	1078:.....N.....Q.....A.....F.....R.....	1177
43526	1157:TPSVGVSYNHLGSTNFKSNTNKVALSNGSSQHLFNASANVEARYTYGDTSYFYHAGYLQEFANFGSSNAYSINTFKVNAARPLNTHPRVYNGGEQF	1256
60190	1154:K.....	1253
Tx.30a	1178:K.....Q.....N.....R.....M.....T.....A.....	1276
43526	1257:LAKEVFLNLGFLVYLNLSMIGFASNLGMYSF	1290
60190	1254:.....V.....	1287
Tx.30a	1277:.....V.....AS.....	1310

Figure 2: Alignment of the deduced *VacA* amino acid sequences between Tox+ strain 43526 (sequenced in this study), Tox+ strain 60190, and Tox- strain Tx30a. The 3873 bp *vacA* ORF encodes 1291 amino acids. Colons denote identity, and asterisks indicate conserved amino acids.

STATISTICAL METHODS

Proportions were compared by Fisher's exact test.

Results

SEQUENCE ANALYSIS OF *vacA* GENE FROM *H. PYLORI* STRAIN 43526

The entire *vacA* homologue from ATCC 43526, a vacuolating cytotoxin positive strain, was cloned (pBS*vacA*) (fig 1) and sequenced. A 3873 bp ORF was present, which encoded 1291 amino acids (fig 2). The predicted molecular weight of the cytotoxin was 138 700, which is similar to the size (139–140 kDa) of *vacA* products encoded by other strains.¹⁴ The amino acid sequences showed a similarity of 88–91% with those of cytotoxin positive strains described previously,^{22–28} but only 74% with that of the Tx30a cytotoxin negative strain.²¹

EXPRESSION OF VACUOLATING CYTOTOXIN IN *E. COLI*

From the plasmid pBS*vacA* containing the entire *H. pylori vacA* gene, the initial 2233 bp of the gene was cloned into the plasmid vector pQE32 downstream of a synthetic inducible

Figure 3: Expression of the *vacA* gene product in *E. coli*. SDS-PAGE with Coomassie brilliant blue staining shows the recombinant *VacA* protein with the expected molecular weight of about 80 kDa (arrow). The migration of protein molecular mass standards is shown.

promoter (fig 1). This construction, pQE32*vacA*, was introduced into *E. coli* JM109, and the expression of the toxin gene was induced by treatment with IPTG (isopropyl-β-D-thiogalactopyranoside). To facilitate purification of the recombinant protein, it was expressed with six amino-terminal histidines (fig 1) which enable purification by nickel chelation chromatography. The recombinant protein was expressed in *E. coli*, and the purified protein showed a single band on SDS-PAGE (fig 3). The protein was purified in 8 M urea, then dialysed stepwise for removal of urea. The protein was inactive in the vacuolation assay with HeLa cells both before and after dialysis (data not shown).

DETECTION OF *VAC A* EXPRESSION BY *H. PYLORI* ISOLATES

The presence of *vacA* proteins in the culture supernatant was tested by western blotting with the rabbit antisera against the recombinant *VacA* protein and VA-1. Antisera against the recombinant *VacA* protein recognised a band of approximately 87–94 kDa that varied slightly in size between Tox+ strain 43526 (fig 4, lane 3), Tox- strain Tx30a (lane 4), and Tox+ clinical isolates (lanes 6 and 7). The vacuolating cytotoxin was not present (lane 8) in the supernatant of the Tox- clinical isolate. Antisera against VA-1 recognised the cytotoxin in the cell lysate of strain 43526 at the same position as it was recognised by antirecombinant *VacA* protein sera (lane 5).

Figure 4: Western blotting analysis of VacA in *H pylori*. Culture supernatants from *H pylori* strains were probed with control rabbit serum (lanes 1 and 2), rabbit serum raised against the recombinant VacA protein expressed in *E coli* (lanes 3–4, 6–8), and rabbit serum raised against VA-1 (lane 5). The size variability of VacA protein in Tox+ strain 43526 (lanes 1, 3, and 5) or Tox– Tx30a (lanes 2 and 4) is shown. The position of protein molecular mass standards is indicated. *H pylori* clinical isolate culture supernatants were also blotted with antirecombinant VacA rabbit sera (lane 6, Tox+; lane 7, Tox–; lane 8, Tox–).

Figure 5: Western blotting with human sera or rabbit anti-VA-1 antiserum of the purified recombinant VacA protein. The recombinant VacA proteins were electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with human sera (lane 2, negative; lane 3, positive) or rabbit antiserum raised against VA-1 (lane 4, control; lane 5, immunised by VA-1). Gold staining of the cellulose membrane to which purified recombinant VacA protein was electroblotted is also shown (lane 1). The migration of protein molecular mass standards is indicated.

DETECTION OF ANTI-VACA ANTIBODY IN HUMAN SERA

Serum samples from 97 patients who had undergone endoscopy were examined by western blotting using recombinant VacA protein (fig 5). *H pylori* infection was determined by conventional methods (culture and rapid urease test) on gastric biopsy specimens, serum anti-*H pylori* IgG test, and PCR amplification of *ureA* gene in gastric juice.²⁵ If more than two of the tests were positive, the patient was regarded as *H pylori* positive. By this criterion, *H pylori* infection was positive in 84 patients, and negative in 13. The serum anti-VacA antibody was positive in 77/84 (92%) *H pylori* positive and 3/13 (23%) negative patients (table 1). In the group of infected patients with gastric ulcer, duodenal ulcer, chronic gastritis, and endoscopically normal mucosa, the serum positive rate against *vacA* protein was 85% (22/26), 95% (20/21), 95% (18/19), and 100% (14/14), respectively (table 2). There was no significant difference in the seropositivity among groups.

TABLE 1 Comparison of anti-recombinant VacA IgG antibody and *H pylori* infection diagnosed by culture, urease test, anti-*H pylori* antibody, or PCR methods in endoscoped patients

		Western blotting to recombinant VacA protein		
		Positive	Negative	Total
<i>H pylori</i> infection	Positive	77	7	84
	Negative	3	10	13
	Total	80	17	97

TABLE 2 Positive rates of anti-recombinant VacA antibody detected by western blotting in *H pylori* infected patients' sera

Diagnosis*	No (%) of patients positive for anti-VacA antibody	Total
Gastric ulcer	22 (84.6)	26
Duodenal ulcer	20 (95.2)	21
Chronic gastritis	18 (94.7)	19
Endoscopically normal	14 (100)	14
Others†	3 (75.0)	4
Total	77 (91.7)	84

*Patients were classified into five groups according to endoscopy findings.

†Including three patients with gastric cancer and a patient with gastric adenoma.

Discussion

The vacuolating cytotoxin is encoded by a 3864 bp ORF in the *vacA* gene.²² The 137 kDa *vacA* gene product consists of three regions: a 33 amino acid amino-terminal signal peptide, a mature cytotoxin domain of approximately 87 kDa, and a carboxy-terminal 50 kDa segment which is cleaved from the molecule during transmembrane export.^{22–27} The recombinant protein described in this study predominantly covers the 87 kDa domain.

Previous studies indicated that the vacuolating cytotoxin, which induces cellular vacuolation in a number of epithelial cell lines in vitro, is produced only by a subset of *H pylori* isolates.^{11–13} Furthermore, several studies suggested that infection by cytotoxin positive strains was correlated with the development of gastroduodenal diseases.^{12–29} Figura *et al*²⁹ reported that 67% of *H pylori* strains isolated from 24 patients with peptic ulcers produced cytotoxin, whereas only 30% of strains from 53 patients with chronic gastritis produced cytotoxin. Fox *et al*¹² reported that the cytotoxin positive *H pylori* strains were isolated from 69% of 26 patients with diffuse antral gastritis and 89% of 79 patients with chronic atrophic gastritis. However, their study groups were rather small in number. Further studies on large numbers of patients with and without gastrointestinal diseases are therefore needed.

Cytotoxicity assays, using culture supernatant and cell lines of previous studies,¹¹ are essential but may not be suitable for the analysis of a large number of cases, since fresh biopsy specimens are necessary for bacterial culture. Several researchers have analysed cytotoxin neutralising activities using patient sera, which presumably represent neutralising antibodies against the toxin.^{16–19} However, the specificity and sensitivity of this neutralising assay are not sufficient. For example, Pereira *et al*¹⁶ detected serum neutralisation in 17/21 Tox+ infected patients and in 9/41 Tox– infected patients. Hirai *et al*²⁰ detected neutralising activity in 10/19 *H pylori* negative patients. Thus the neutralising method is not sufficient to elucidate the relation between vacuolating cytotoxin and gastroduodenal diseases in detail. Furthermore, the biological assay is complex and easily affected by many factors. We therefore tried to develop a simpler and more sensitive assay system.

In the present study, the *vacA* gene was cloned to produce a recombinant cytotoxin protein in large amounts. The recombinant

protein was easily purified due to its histidine tag.³¹ Using this protein, antibodies which specifically react with the vacuolating cytotoxin in bacterial culture supernatant were successfully raised, demonstrating the intact immunogenicity of the recombinant cytotoxin. The size of the native cytotoxin, which reacted with the antibody against the recombinant cytotoxin, was the same as that which reacted with the antisera against VA-1, the antibody raised in rabbits. We suggest that both antibodies react with the native vacuolating cytotoxin. The recombinant and native cytotoxins are thus thought to have a common epitope. Using the recombinant protein, assessment of specific antibodies against the cytotoxin in patient sera was possible.

The recombinant protein purified in the present study failed to induce HeLa cell vacuolation in vitro, as reported previously.³² This may be due to the quaternary structure of active vacuolating cytotoxin, which is comprised of the multiple cytotoxin subunits, and failure of the recombinant cytotoxin to assemble correctly into an oligomeric structure.^{26, 32}

The western blot assay using the purified recombinant vacuolating cytotoxin revealed the presence of antivacuolating cytotoxin antibodies in sera of the majority of patients with *H. pylori* infection, regardless of the type of gastroduodenal disease (peptic ulcer, chronic gastritis, and endoscopically normal mucosa). Chronic atrophic gastritis was observed endoscopically in 62/84 (74%) *H. pylori* positive patients and no relation was found between antivacuolating cytotoxin antibody and the presence of chronic atrophic gastritis. These results suggest that most strains of *H. pylori* in Japan produce the vacuolating cytotoxin in vivo, differing from previous results of others.^{11-13, 16-20}

There is a possibility that our results might differ from others due to use of different methods. The western blot method incorporating recombinant protein for detecting antibody to the cytotoxin may lack specificity. For example, antirecombinant VacA IgG antibody was observed in serum from 77/84 (92%) infected patients (table 1) and this prevalence is higher than has been reported previously in other studies, which used different methods. Similarly, antibody was detected in 3/13 uninfected patients (table 1). In addition, the western blot procedure detects antibody against the "inactive" cytotoxin of strain Tx30a (fig 4) and the existence of inactive, but secreted cytotoxin was reported previously.²¹⁻³³ Each of these three items suggests at least the potential for poor specificity.

However, Xiang *et al.*³³ have reported that the protein is produced in only 2/16 Tox- *H. pylori* strains, and Tox- strains which produce cytotoxin without vacuolating activity are believed to be uncommon. Thus, by serum anticytotoxin antibody analysis, the prevalence of cytotoxin positive strains may be estimated. In fact, most *H. pylori* isolates (34/38 strains) produce the active cytotoxin in vitro (unpublished observation), and vacuolation assay revealed that the majority of the strains have

active cytotoxin in a subset of the patients in this study (data not shown). In addition, we have reported that the signal sequence of *vacA* gene is type S1, indicative of Tox+ strains, in all 28 *H. pylori* strains isolated from Japanese patients.^{21, 34} These data may confirm the results in the present study.

Gastric cancer remains one of the most frequently diagnosed malignancies worldwide, and Japan has a high incidence of gastric malignancies, with 38.2 deaths per 100 000 people in 1993.³⁵ Epidemiological studies have demonstrated that the prevalence of *H. pylori* infection is significantly higher in patients with gastric cancer than in the general population.³⁶ However, the mechanism by which *H. pylori* causes gastric cancer is not known. Our results showed that the majority of *H. pylori* strains from Japanese patients were positive for the vacuolating cytotoxin; it may therefore be conceivable that the high incidence of gastric cancer in Japan is related not only to the high prevalence of *H. pylori* infection but also to the high rate of cytotoxin producing strains in our country. A prospective large scale study using this western blot assay might reveal the relation between the incidence of gastric cancer and infection with cytotoxin positive *H. pylori* strains. Furthermore, other proteins produced by *H. pylori* should also be investigated—the size of the *H. pylori* genome suggests that it produces about 1700 proteins.³⁷

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