Molecular mimicry by *Helicobacter pylori* CagA protein may be involved in the pathogenesis of *H. pylori*-associated chronic idiopathic thrombocytopenic purpura

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Summary

The eradication of *Helicobacter pylori* often leads to platelet recovery in patients with chronic idiopathic thrombocytopenic purpura (cITP). Although this clinical observation suggests the involvement of *H. pylori*, little is known about the pathogenesis of cITP. We initially examined the effect of *H. pylori* eradication on platelet counts in 20 adult Japanese cITP patients. Then, using platelet eluates as the probe in immunoblot analyses, we examined the role of molecular mimicry in the pathogenesis of cITP. *Helicobacter pylori* infection was detected in 75% (15 of 20) of cITP patients. Eradication was achieved in 13 (87%) of the *H. pylori*-positive patients, seven (54%) of which showed increased platelet counts within the 4 months following treatment. Completely responsive patients also showed significant declines in platelet-associated immunoglobulin G (PAIgG) levels. Platelet eluates from 12 (nine *H. pylori*-positive and three *H. pylori*-negative) patients recognized *H. pylori* cytotoxin-associated gene A (CagA) protein, and in three completely responsive patients, levels of anti-CagA antibody in platelet eluates declined after eradication therapy. Cross-reactivity between PAIgG and *H. pylori* CagA protein suggests that molecular mimicry by CagA plays a key role in the pathogenesis of a subset of cITP patients.

Keywords: chronic idiopathic thrombocytopenic purpura, *Helicobacter pylori*, molecular mimicry, cytotoxin-associated gene A protein.

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Idiopathic thrombocytopenic purpura (ITP) is an acquired autoimmune disorder characterized by a low platelet count and mucocutaneous bleeding. The cause is destruction of platelets in the reticuloendothelial system mediated by platelet-bound autoantibodies (Cines & Blanchette, 2002). The targets of the anti-platelet autoantibodies include a variety of platelet proteins, among them glycoproteins IIb/IIIa, Ib/IX, Ia/IIa and IV. Several groups have recently reported that eradication of *Helicobacter pylori* leads to platelet recovery in patients with chronic ITP (cITP) (Gasbarrini *et al*, 1998; Grimaz *et al*, 1999; Tohda & Ohkusa, 2000; Emilia *et al*, 2001; Kohda *et al*, 2002; Veneri *et al*, 2002). Although these clinical observations suggest the involvement of *H. pylori*, little is known about the mechanisms responsible for triggering production of the anti-platelet autoantibodies involved in the pathogenesis of cITP.

The gram-negative bacterium *H. pylori* is the human pathogen responsible for chronic gastritis and peptic ulcers; moreover, infection with this organism also increases the risk of gastric cancer and mucosa-associated lymphoid tissue lymphoma (Suerbaum & Michetti, 2002). During the gastritis,

H. pylori infection induces production of anti-gastric epithelial autoantibodies through a process of molecular mimicry involving the gastric epithelium and one or more *H. pylori* antigens (Negrini *et al*, 1996). Notably, *H. pylori* has also been implicated in the pathogenesis of some autoimmune diseases, such as rheumatoid arthritis, autoimmune thyroiditis and Sjögren's syndrome (Gasbarrini & Franceschi, 1999). In the present study, we therefore examined the effect of *H. pylori* eradication in a group of Japanese cITP patients with the aim of understanding better the role an autoimmune response mediated by molecular mimicry in the pathogenesis of cITP.

Patients and methods

Patients

Twenty adult cITP Japanese patients (five men and 15 women; mean age, 53 years) with cITP were enrolled prospectively. ITP was defined by thrombocytopenia (platelet count $<120 \times 10^9$/l) without megakaryocytic hypoplasia in the bone marrow, and by exclusion of other causes. Patients with secondary autoimmune thrombocytopenia were excluded. Platelet-associated immunoglobulin G (PAIgG) levels were determined using an enzyme-linked immunosorbent assay (normal range: 9·0–25·0 ng/10^7 platelets).

^{13}C-urea breath tests (Otsuka, Tokyo, Japan) were used to diagnose *H. pylori* infection. Eradication of *H. pylori* was assessed 8 weeks after treatment using the same test. The clinical responses to *H. pylori* eradication were evaluated 4 months after treatment: a complete response was defined as an increase in the platelet count to more than 120×10^9/l; a partial response was defined as an increase $>20 \times 10^9$/l above pretreatment platelet counts.

The regimen for *H. pylori* eradication, which entailed administration of clarithromycin (400 mg twice daily), amoxicillin (1500 mg twice daily) and lansoprazole (60 mg twice daily) for 7 d, was administered to both *H. pylori*-positive and -negative patients. During the study period, no other new therapies for ITP were added, although patients who were receiving maintenance therapy for ITP continued to do so with no changes.

This study was approved by the Institutional Review Board of Yamaguchi University Hospital; informed consent was obtained from all participants according to the terms of the Declaration of Helsinki.

Platelet eluates

Platelets were harvested from 40 ml of whole blood in ethylenediaminetetraacetic acid (EDTA), washed four times with phosphate-buffered saline (PBS) containing 2% EDTA and 15% acid-citrate-dextrose, and resuspended in PBS containing 0·2% bovine serum albumin (BSA). PAIgG was eluted from 5×10^7 washed platelets using ether according to the method of von dem Borne *et al* (1980), after which the eluates were stored at −20°C until use.

Cell lysates from H. pylori

Helicobacter pylori (NCTC11637) was cultured in Brucella-broth as described previously (Okamoto *et al*, 2002). To obtain cell lysates, the cells were washed twice with 50 mmol/l PBS and suspended in 10 mmol/l PBS, after which they were incubated with lysozyme for 20 min at room temperature, sonicated, and centrifuged at 8000 × g for 15 min at 4°C. The resultant supernatant was collected as the cell lysate.

Immunoblot analysis

Helicobacter pylori cell lysates were subjected to 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) containing 0·1% Tween 20 (TBS-T) and then incubated for 12 h at 4°C with platelet eluates from 5×10^7 washed platelets in 4 ml of TBS containing 3% BSA. After washing the labelled membranes with TBS-T, they were incubated for 1 h with horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) polyclonal antibodies (Jackson ImmunoResearch, West Grove, PA, USA) and visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA).

Immunoprecipitation

Samples of cell lysate containing 1 mg of *H. pylori* protein in 500 μl of PBS were incubated for 2 h at 4°C with 2 μg of goat anti-cytotoxin-associated gene A (CagA) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), after which immunoprecipitation was facilitated with protein G-Sepharose (Amersham Biosciences, Piscataway, NJ, USA). The immune complexes were separated by SDS-PAGE, transferred to nitrocellulose membranes, and analysed by immunoblotting with platelet eluates.

Statistical analysis

Differences between *H. pylori*-positive and *H. pylori*-negative patients with respect to age, gender, disease duration and platelet count were analysed using non-parametric Mann–Whitney *U*-tests, as were differences in PAIgG levels in responding and non-responding patients before treatment. PAIgG levels before and after treatment were compared using the Wilcoxon signed-rank sum test. *P*-values $<0·05$ were considered significant.

Results

Patient characteristics and outcome

The characteristics and outcomes of all 20 cITP patients studied are shown in Table I. Before treatment, both infected and uninfected patients were PAIgG-positive and showed no

Table I. Characteristics and outcomes of patients.

Patient*	Age (years)	Sex	Contraction period (months)	Previous treatment†		Eradication	Platelet count‡			PAIgG (ng/10^7plts)		Response§
				Therapy	Response§		Before	1 month	4 month	Before	4 month	
P1	48	F	72	PSN, S	NR	Yes	36	183	134	87·9	30·2	CR
P2	64	F	126	–	–	Yes	57	114	127	54·7	60·2	CR
P3	70	F	120	PSN, K	NR	Yes	17	137	211	46·7	23·8	CR
P4	67	M	25	–	–	Yes	76	156	196	29·8	26	CR
P5	46	F	12	PSN	PR	Yes	27	229	276	59·3	22·2	CR
P6	17	F	35	PSN	PR	Yes	66	220	198	53·1	20·3	CR
P7	61	M	37	–	–	Yes	33	78	80	137·7	106·9	PR
P8	54	M	108	–	–	Yes	18	16	13	297·9	411·7	NR
P9	44	F	106	PSN, S	NR	Yes	6	6	2	1006·1	853·1	NR
P10	37	F	24	–	–	Yes	103	95	106	64·4	34·8	NR
P11	62	F	156	PSN	NR	Yes	10	22	26	241	103·6	NR
P12	52	F	108	PSN	NR	Yes	45	45	44	218	125·6	NR
P13	61	F	23	PSN	PR	Yes	49	52	29	71·8	209·7	NR
P14	49	F	180	PSN	NR	No	32	30	28	74·6	54·8	NR
P15	63	M	60	–	–	No	23	30	46	70·8	89·9	PR
N1	28	F	27	PSN, Ig, K	NR		12	15	20	520	142·5	NR
N2	26	F	84	PSN	PR		111	117	83	65·9	114·2	NR
N3	55	M	216	–	–		8	10	8	172·1	212·8	NR
N4	64	F	181	PSN, S	NR		23	22	24	45·6	61·4	NR
N5	57	F	252	PSN	PR		42	43	42	250·5	623·7	NR

*P and N indicate positive and negative for *H. pylori* infection respectively.
†PSN, prednisolone; S, splenectomy; Ig, intravenous immunoglobulin; K, kami-kihito herbal medicine.
‡Platelet counts ($\times 10^9$/l) before, 1 month and 4 months after eradication.
§CR, complete response; PR, partial response; NR, no response.

significant differences with respect to age, gender, disease duration or platelet count. The prevalence of *H. pylori* infection was 75% (15 of 20), and bacterial eradication was achieved in 13 (87%) of the *H. pylori*-positive patients. Four months after treatment, 7 (54%) of the patients in whom *H. pylori* had been eradicated showed increased platelet counts. Of those, six responded completely and one showed a partial response. In addition, one patient (P15) in whom eradication was not achieved also showed a partial response. Despite our failure to eradicate *H. pylori* in that patient, the urea breath test levels decreased after treatment. All patients who responded completely showed recovery of platelet counts within 1 month after eradication. By contrast, none of the five *H. pylori*-negative patients showed improvement in their platelet counts, despite receiving the same treatment regimen.

The PAIgG levels also declined significantly in responding patients following *H. pylori* eradication; no significant decline was observed in non-responding patients (Table II). Notably, the pretreatment levels of PAIgG were significantly lower in responding patients than in non-responding ones ($P = 0{\cdot}016$, Table II).

Table II. Comparison of PAIgG levels.

	PAIgG (mean ± SEM, ng/10^7 platelets)		
	Before treatment	After treatment	*P*-value
Responding patients ($n = 6$)	67·0 ± 13·5	41·4 ± 12·1	0·04
Non-responding patients ($n = 14$)	252·3 ± 79·1	245·7 ± 73·8	0·87

H. pylori immunoblot analysis

The presence of antibodies recognizing both platelets and *H. pylori* antigens was evaluated by subjecting platelet eluates from 18 cITP patients to Western blot analysis. Because of severe thrombocytopenia or platelet aggregation, sufficient platelets for analysis could not be obtained from two patients (P9 and P11). Platelet eluates from 12 (nine *H. pylori*-positive and three *H. pylori*-negative) patients recognized one or more *H. pylori* proteins, and all 12 eluates recognized a single 140 kDa *H. pylori* protein (Fig 1A). Contamination of the platelet eluates by serum IgG was excluded, as none of the eluates from three *H. pylori*-positive non-thrombocytopenic volunteers recognized any *H. pylori* proteins (Fig 1B).

The CagA antigen is a highly antigenic *H. pylori* protein. Its molecular weight,140 kDa (Fig 1C, lane 1), suggested that it might be the protein recognized by the platelet eluates from

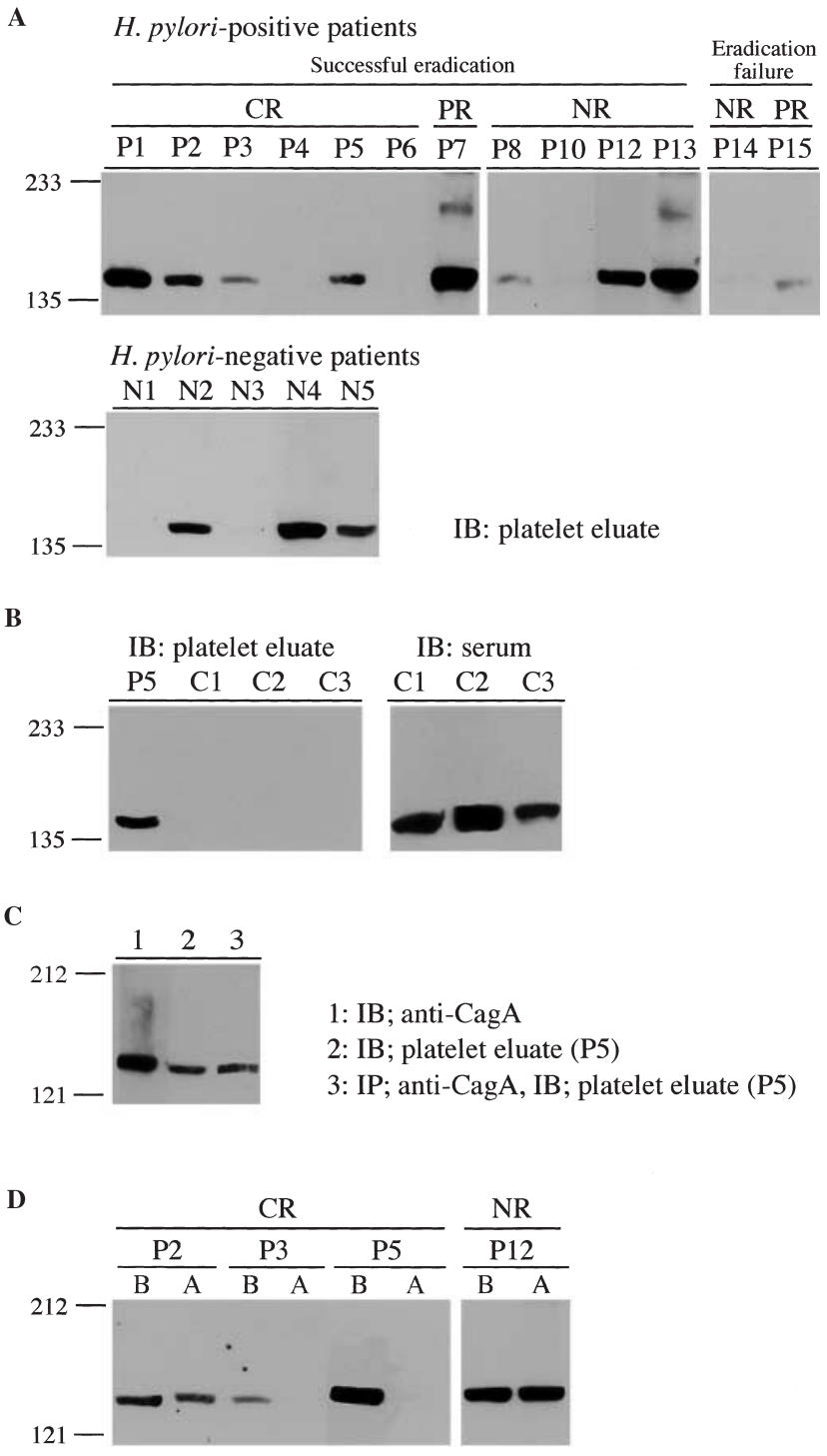

Fig 1. Immunoblot and immunoprecipitation assays. (A) *Helicobacter pylori* proteins were separated by SDS-PAGE and immunoblotted with platelet eluates (PAIgG) from *H. pylori*-positive (P1-P15) and *H. pylori*-negative (N1-N5) cITP patients. (B) *H. pylori* proteins were also immunoblotted with platelet eluates and serum from *H. pylori*-positive non-thrombocytopenic volunteers (C1-C3). P5 is shown as a positive control. (C) *H. pylori* proteins were immunoblotted with anti-CagA antibody (lane 1) or platelet eluate from a *H. pylori*-positive cITP patient (P5) (lane 2). In lane 3, total *H. pylori* lysate was subjected to immunoprecipitation with anti-CagA antibody and then immunoblotted with platelet eluate from P5. (D) Comparison of the levels of anti-CagA antibody present in platelet eluates before and after *H. pylori* eradication. *H. pylori* proteins were immunoblotted with platelet eluates from completely responsive patients (P2, P3, and P5) and one unresponsive patient in whom eradication was successful (P12). B and A, before and after eradication respectively: CR, complete response; PR, partial response; NR, no response; IB, immunoblot; IP, immunoprecipitation. Numbers on the left indicate molecular weights in kDa. For all immunoblot analyses, eluates from 5×10^7 platelets were used as primary antibodies.

cITP patients (e.g., P5) (Fig 1C, lanes 1, 2). This idea was substantiated when the CagA protein that was immunoprecipitated using a specific anti-CagA antibody was recognized by platelet eluate (Fig 1C, lane 3). Furthermore, levels of anti-CagA antibody decreased in platelet eluates from three patients who responded completely to eradication therapy (P2, P3 and P5). By contrast, no reduction in anti-CagA antibody was observed in the eluate from a non-responding patient (P12), although the *H. pylori* was successfully eradicated (Fig 1D).

Discussion

Several clinical studies have demonstrated a beneficial effect of *H. pylori* eradication on platelet recovery in cITP patients

(Gasbarrini *et al*, 1998; Emilia *et al*, 2001; Kohda *et al*, 2002; Veneri *et al*, 2002) and a similarly good response rate (54%) was achieved in the present study. Moreover, by treating *H. pylori*-negative patients with the same regimen of antibiotics, we showed that the drugs themselves exerted no direct pharmacological effects leading to improved platelet counts, which confirmed that the efficacy of the treatment was eradication-dependent and therefore limited to *H. pylori*-positive cITP patients. Thus, to relieve the autoimmunity seen in patients with *H. pylori*-associated cITP, it is essential to eliminate the persistent infection.

There are a variety of infectious organisms that express molecular mimic antigens involved in the pathogenesis of autoimmune diseases (Wucherpfennig, 2001) – e.g. rheumatic fever, Guillain-Barré syndrome (Yuki *et al*, 1993) and immune thrombocytopenia (Bettaieb *et al*, 1992; Bettaieb *et al*, 1996). Among these, *H. pylori* induces production of anti-gastric epithelium autoantibodies (Claeys *et al*, 1998); moreover, monoclonal antibodies against *H. pylori* reportedly cross-react with several other human tissues, including salivary gland, renal tubular epithelium and duodenal epithelium (Ko *et al*., 1997). We have shown here that PAIgG from several cITP patients recognized *H. pylori* CagA protein and that cross-reactive antibody levels decreased following *H. pylori* eradication in patients that showed a complete response. This is consistent with the idea that *H. pylori* infection exerts a causative effect on the autoimmunity responsible for cITP via molecular mimicry. In addition, the finding that platelet eluates from three of five *H. pylori*-negative cITP patients also recognized CagA suggests that the anti-CagA antibody present in the eluate is the anti-platelet autoantibody produced in cITP, and is not an anti-*H. pylori* antibody produced during normal immune responses to bacterial infection.

Molecular mimicry is one way to break immunological tolerance and initiate the production of autoantibodies. Normally, autoreactive B cells cannot produce autoantibodies because they receive no help from autoreactive $CD4^+$ T cells, which are functionally deleted. However, if a cross-reacting non-self antigen is encountered, the B cells can present peptides from this molecule to non-self reactive $CD4^+$ T cells, thereby driving them to produce autoantibodies (Roitt *et al*, 1998). We suggest that *H. pylori* CagA may be such a cross-reactive antigen. Anti-platelet autoreactive B cells recognize CagA and can present it to *H. pylori*-reactive $CD4^+$ T cells under conditions of persistent infection. B cells may also produce anti-platelet autoantibodies without the help of autoreactive $CD4^+$ T cells in a subset of cITP patients. Enzyme-linked immunoSPOT assays have recently been used to evaluate these autoreactive anti-platelet B cells (Kuwana *et al*, 2002). Although we could use this approach in the present study, it may be useful in future studies for further analysis of the pathogenesis of *H. pylori*-associated cITP.

The good platelet recovery achieved after eradication of *H. pylori* in the present study is consistent with earlier studies from Japan and Italy (Gasbarrini *et al*, 1998; Emilia *et al*, 2001; Kohda *et al*, 2002; Veneri *et al*, 2002). On the other hand, a Spanish group reported lower response rates (13%), although the prevalence of *H. pylori* infection does not differ among ethnic groups (Jarque *et al*, 2001). This probably reflects the fact that some *H. pylori* strains do not harbour the CagA gene, and that CagA-positivity varies depending upon the geographic location (Perez-Perez *et al*, 1997; Mobley *et al*, 2001). In Japan, most *H. pylori* strains do harbour the CagA gene (Maeda *et al*, 1997), which probably accounts for the efficacy of eradication therapy in the treatment of cITP there. Recently, a French group reported no reactivity between platelet eluates and *H. pylori* proteins in ITP (Michel *et al*, 2002). We think the apparent absence of cross-reactivity might be because of the small number of patients examined in that study or the low incidence of CagA-positivity in France.

Unfortunately, because the amounts of PAIgG available from individual patients were very limited, we were unable to determine the platelet antigens that PAIgG recognizes in common with CagA protein. In addition, cross-reactivity between platelet antigens and *H. pylori* antigens other than CagA may also be involved in the pathogenesis of *H. pylori*-associated cITP, as the platelet eluates from two completely responsive patients (P4 and P6) did not recognize CagA protein.

In summary, we have demonstrated cross-reactivity between PAIgG and *H. pylori* CagA protein and suggest that molecular mimicry by CagA of an unknown platelet antigen is crucially involved in the pathogenesis of a subset of cITP cases. Further investigation should enable identification of the platelet antigen that shares an epitope with CagA, as well as clarification of the host susceptibility factors via which autoimmunity is induced.

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