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Association between *Helicobacter pylori* infection and platelet count in mice

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Abstract: Strong evidence for an association between idiopathic thrombocytopenic purpura (ITP) and *Helicobacter pylori* (HP) infection has been reported in humans. Chronic ITP is known to be improved by the eradication of HP. The purpose of this study was to reproduce these events by the experimental infection of several strains of mice with HP. BALB/c, C57BL/6, and DBA/2 mice were untreated or orally inoculated with HP. Two months later, platelet counts were compared in samples from HP-infected and noninfected mice. Platelet counts (mean ± SD, × 10^4 cells/μl) in blood samples from HP-infected BALB/c, C57BL/6, and DBA/2 mice were 102.28 ± 14.71, 99.65 ± 17.00, and 111.57 ± 16.20, respectively; the respective counts from noninfected mice were 121.80 ± 13.30, 104.35 ± 18.20, and 107.84 ± 14.33. A significant difference in platelet counts between HP-infected and noninfected mice was observed in BALB/c mice ($P \leq 0.01$) but was not observed in DBA/2 mice, even though the histocompatibility (H)-2 type of the DBA/2 was the same as that of BALB/c mice. According to ELISA results, the optical density value for the anti-HP antibody in HP-infected BALB/c mice was not correlated with the number of platelets ($P > 0.50$). These results suggest that the decrease in platelet count caused by HP infection is not related to antibody titer and histocompatibility-2 type. Experimental infection of BALB/c mice with HP can reproduce the relationship between HP and ITP and serves as a good model to investigate the mechanistic basis for the effectiveness of HP eradication therapy for ITP treatment.
Key words: *Helicobacter pylori*, infection, ITP, mouse, strain

Introduction

Idiopathic thrombocytopenic purpura (ITP) is an acquired bleeding disorder caused by autoantibody-mediated platelet destruction and impaired thrombopoiesis [1]. ITP is characterized by a low platelet count (<1 × 10^{11} cells/l) and mucocutaneous bleeding [15, 20]. Cytotoxic T cells and antiplatelet antibodies also contribute to the pathogenesis of ITP [19]. Platelet destruction is mediated by the IgG fraction of the reticuloendothelial system [3], although the mechanistic details are not fully understood.

Major histocompatibility complex (MHC) class I human leukocyte antigen *(HLA)-B7*-associated peptides identified as glycoprotein Ib have been detected on the platelet membrane in healthy individuals and ITP patients [11]. However, there is no clear evidence of an association between ITP and specific MHC class I or II polymorphism [11, 17, 18].

The incidence of ITP is estimated to be three to four

out of 10^5 individuals per year and varies depending on age, sex [14], obesity [10], and pathogen infection or vaccination status [7, 16]. Autoimmune manifestations, including ITP, have been reported following vaccination against several infectious agents, including measles, mumps, and rubella, *Pneumococcus*, *Haemophilus influenza* type B, hepatitis B virus, and varicella-zoster virus [4, 8, 17, 21].

Helicobacter pylori (HP) infection has been linked to ITP. HP eradication improves chronic ITP [12]. HP induces antibodies that cross-react with platelets [22]. Molecular mimicry by the HP CagA protein was reported to play a key role in the development of ITP in a subset of chronic patients [5]. However, little is known about the mechanisms underlying antiplatelet autoantibody production in the pathogenesis of chronic ITP.

To address this point, in the present study, we established mouse ITP models that recapitulate the relationship between HP infection and platelet count and used the models to investigate this association in the context of two different types of histocompatibility *(H)-2*, *H-2^b* haplotypes (C57BL/6NCrl [B6] mice) and *H-2^d* haplotypes (BALB/cAnNCrlCrlj [BALB] and DBA/2NCrl [D2] mice).

Materials and Methods

Animals

Fifty-six male mice (aged 3 weeks) including 19 BALB, 20 B6, and 17 D2 mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). The mice were housed under standard conditions at a controlled temperature ($24 \pm 2°C$) and relative humidity ($50 \pm 5\%$) on a 12:12-h light/dark cycle and were supplied food and water *ad libitum*. The study was carried out in accordance with a protocol approved by the Ethics Committee for Animal Experiments of Teikyo University.

Experimental HP infection and sampling

HP (SS1 strain) [13] was grown on HP-selective agar (Eiken Chemical Co., Ltd.,Tokyo, Japan) for 7 days at 37°C in a microaerophilic environment using an AnaeroPack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). Colonies were picked from the plates and resuspended in tryptic soy broth (BD, Tokyo, Japan) as an inoculant. Mice in each strain were divided into two groups. The first group (BALB, n=9; B6, n=10; and D2,

n=7, aged four weeks) was orally inoculated with 1.0–3.7 $\times 10^7$ colony-forming units of bacteria three times over 3 days. The second group of mice (n=10 of each strain) was inoculated with broth instead of the bacterial solution. Two months after inoculation, blood and stomach tissue samples were collected. Blood samples from axillary arteries and veins of mice were collected in ethylenediaminetetraacetic acid (EDTA), and plasma samples were prepared for antibody testing. The stomach tissue samples were collected for PCR and histopathological tests.

Detection of anti-HP antibody by enzyme-linked immunosorbent assay(ELISA)

The wells of a 96-well plate were coated with HP antigen (1.0 μg) in carbonate and bicarbonate buffer, followed by overnight incubation at 4°C. The wells were washed with phosphate-buffered saline (PBS), and 1% bovine serum albumin was added to block uncoated sites by overnight incubation at 4°C. The wells were washed as above, and 200 μl of plasma samples diluted 1:40 was added to individual wells followed by incubation at 37°C for 1 h. The wells were washed, and 200 μl of horseradish peroxidase-conjugated Protein A was added to each well, followed by incubation for 1 h at 37°C. The wells were washed with PBS, and 200 μl freshly prepared solution containing o-phenylenediamine dihydrochloride and H_2O_2 as substrates was added to each well, followed by incubation in the dark for 10 min at 37°C. The enzyme-substrate reaction was halted by adding 50 μl of 1 M H_2SO_4. The optical density of each sample at a wavelength of 490 nm (OD_{490}) was measured using a VersaMax Microplate Reader (Molecular Devices Japan, Tokyo, Japan).

Detection of HP DNA in stomach tissue by PCR

DNA was extracted from stomach tissue samples using a MagPrep Bacterial Genomic DNA Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. HP DNA was detected by nested PCR, as previously described [9]. Briefly, template DNA was amplified in a final volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 mM each dNTP, 10 μM primers (5′-CTA TGA CGG GTA TCC GGC-3′, forward, and 5′-CTC ACG ACA CGAGCT GAC-3′, reverse), and 5 U Taq DNA polymerase (Takara Bio). The cycling conditions for the first and second (nested) steps were 94°C for 3 min; 25 cycles of 94°C

Table 1. Comparison of mean OD values for antibodies and blood cell counts between *Helicobacter pylori*-infected and noninfected mice

Strains of mice	Number of mice tested	HP inoculation	PCR for detection of HP Number of positive samples	ELISA antibody against HP Mean of OD_{490} value $\pm$ SD	P value*	Platelet count ($\times 10^4$ cells/μl) Mean $\pm$ SD	P value*	Red blood cells ($\times 10^4$ cells/μl) Mean $\pm$ SD	P value*
BALB/c	9	+	8	0.57 $\pm$ 0.28	0.00	102.28 $\pm$ 14.71	0.01	1,068.22 $\pm$ 143.43	0.67
	10	−	ND**	0.12 $\pm$ 0.02		121.80 $\pm$ 13.30		1,092.90 $\pm$ 95.40	
C57BL/6	10	+	10	0.60 $\pm$ 0.47	0.01	99.65 $\pm$ 17.00	0.56	957.90 $\pm$ 67.34	0.41
	10	−	ND**	0.12 $\pm$ 0.02		104.35 $\pm$ 18.20		989.60 $\pm$ 97.28	
DBA/2	7	+	6	0.83 $\pm$ 1.01	0.05	111.57 $\pm$ 16.20	0.62	1,110.71 $\pm$ 108.30	0.05
	10	−	ND**	0.13 $\pm$ 0.04		107.84 $\pm$ 14.33		1,003.20 $\pm$ 43.12	

*Results were compared with samples from HP infected and noninfected mice. **ND: Not done. ELISA, enzyme-linked immunosorbent assay; HP, *Helicobacter pylori*; OD_{490}, optical density at 490 nm; PCR, polymerase chain reaction; SD, standard deviation.

for 30 s, 55°C for 1 min, and 72°C for 1 min; and 72°C for 10 min. A 1-μl volume of the initial PCR product was used for the second round of amplification using the primers 5′-GTT GGA GGG CTT AGT CTC T-3′ (forward) and 5′-TTA GAG TTC TCA GCA TAA CCT-3′ (reverse). The length of the amplified product was estimated to be 328 bp.

Histopathology

Mouse stomach tissue samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, and cut into 5-μm-thick sections that were stained with hematoxylin and eosin.

Manual counting of platelets

Whole blood samples were diluted with 1% ammonium oxalate solution. The isotonic balance of the diluent was such that all erythrocytes were lysed while the platelets remained intact. The dilution was thoroughly mixed and incubated at 25°C for 15 min to allow lysis of erythrocytes. After incubation, the diluted solution was mounted on a hematocytometer (Erma Inc., Tokyo, Japan). The cells were allowed to settle, and those in a specific area of the hemocytometer chamber were counted using a model E200 microscope (E200, Nikon, Tokyo, Japan).

Manual counting of red blood cells

Whole blood samples in EDTA were diluted with an isotonic solution (Gower's solution), and the diluted blood was introduced into the counting chamber where the cell count was performed microscopically.

Statistical analysis

Data were analyzed using the IBM SPSS v.22 software (IBM Japan, Tokyo, Japan). The significance of differences between manual and automated counts was evaluated with Student's *t*-test. *P* values ≤0.05 were considered statistically significant. Correlations between the test results were evaluated with Pearson's correlation coefficient.

Results

The results of all experiments are shown in Table 1.

Detection of HP DNA in stomach tissue samples using PCR

HP DNA was detected in 8/9, 10/10, and 6/7 stomach tissue samples from BALB, B6, and D2 mice, respectively.

Detection of anti-HP antibodies

The mean $\pm$ SD OD_{490} values for anti-HP antibody in serum detected by ELISA were 0.57 $\pm$ 0.28, 0.60 $\pm$ 0.47, and 0.83 $\pm$ 1.01 in HP-infected BALB, B6, and D2 mice, respectively, as compared with values of 0.12 $\pm$ 0.02, 0.12 $\pm$ 0.02, and 0.13 $\pm$ 0.04 for the respective noninfected counterparts (*P*≤0.05 for all strains).

Histopathological analysis

No lesions were observed in stomach tissue samples, except in a sample from an HP-infected B6 mouse, which had an ulcer at the proventricular boundary that was permeated with inflammatory generative cells (Fig. 1). The mouse that showed the ulcer was positive for HP DNA as assessed by PCR, had an OD_{490} value of 1.22 for anti-HP antibody, and had a platelet count of 84.50 $\times 10^4$ cells/μl.

Fig. 1. An ulcer with inflammatory generative cells permeating the proventricular boundary in the mouse. The mouse was positive for the PCR test, the OD value of the antibody was 1.22, and the number of platelets was 84.50×10^4 cells/μl.

Fig. 2. Correlation between OD_{490} values for detection of antibodies against HP and platelet counts ($\times 10^4$ cells/μl) of HP-infected BALB mice (n=9). Correlation coefficient: r=0.188, *P*=0.629. Regression equation between absorbance (Y) and platelet count (X): $Y = 0.21 + 3.54 \times 10^{-3}X$). Dots in the figure represent individuals.

Platelet count

The mean ± SD platelet counts ($\times 10^4$ cells) in 1 μl of blood samples from HP-infected BALB, B6, and D2 mice were 102.28 ± 14.71, 99.65 ± 17.00, and 111.57 ± 16.20, respectively. In noninfected mice, the counts were 121.80 ± 13.30, 104.35 ± 18.20, and 107.84 ± 14.33, respectively. Only the counts in the BALB strain showed a statistically significant difference between infected and noninfected mice ($P \leq 0.01$). The correlation coefficient between the OD_{490} for anti-HP antibody and the platelet count of HP-infected BALB mice (n=9) was r=0.188 (P=0.629; Fig. 2).

Red blood cell count

The mean ± SD values for the red blood cell counts ($\times 10^4$ cells) in 1 μl of blood samples from HP-infected BALB, B6, and D2 mice were 1,068.22 ± 143.43, 957.90 ± 67.34, and 1,110.71 ± 108.30, respectively. In noninfected mice, the counts were 1,092.90 ± 95.04, 989.60 ± 97.28, and 1,003.20 ± 43.12, respectively. Only the counts in the D2 strain showed a statistically significant difference between infected and noninfected mice ($P \leq 0.05$).

Discussion

In this study, the platelet counts in BALB mice were significantly decreased two months after experimental infection. ITP is 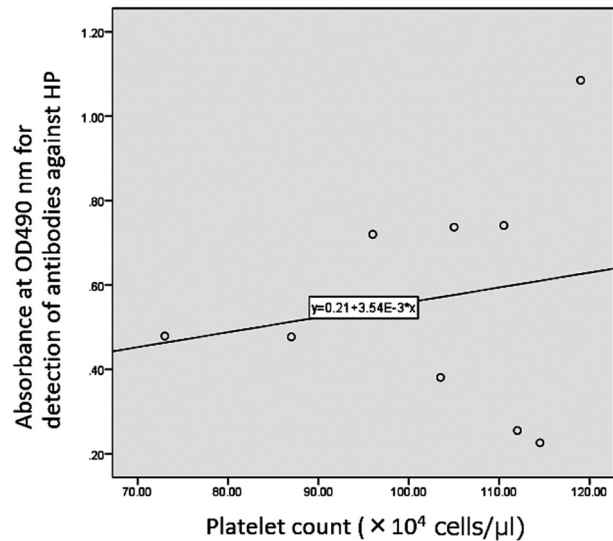an autoimmune disorder caused by in-creased platelet clearance by anti-platelet autoantibodies [6]. HP infection is associated with low platelet counts in ITP patients [8]. Even when HP is eradicated in HP-positive ITP patients, not all patients show improved platelet counts. One possible reason for this is that the anti-HP antibody cross-reacts with platelet surface antigens [15]. However, the results of the present study show that the anti-HP antibody titer (reflected by the OD_{490} value) is not related to the platelet count, suggesting that other factors are involved. The platelet recovery observed in ITP patients after HP eradication therapy could be due to a shift in the monocyte Fcγ receptor balance towards the inhibitory Rfγ receptor IIB (FcγRIIB) [2]. This hypothesis can be tested using our experimental HP model.

In this study, the average platelet count of HP-infected B6 mice was lower than that of noninfected B6 mice; however, this difference was not significant (P=0.56). Moreover, the platelet count was not decreased in D2 mice even upon HP infection. The standard haplotypes are defined by private K and D alleles. BALB and D2 mice share the same H-2 haplotypes (H-$2K^d$ and H-$2D^d$), whereas B6 mice show different haplotypes (H-$2D^b$ and H-$2K^b$). HP-infected BALB mice, but not D2 mice, showed a significant decrease in the platelet count. These results suggest that the H-2 type is not related to the

effects of HP eradication in this study using mice. An HLA-DQB1*03 pattern is associated with a higher probability of platelet response to HP eradication therapy [24]. Our data suggest that MHC polymorphism may be only one of the many factors that mediate this effect.

PCR analysis revealed that the stomach of mice was colonized by HP. However, this was not associated with any lesions, such as gastritis or ulcers, except in one B6 mouse. Host-specific effects, including the type of pathology induced, have been observed according to the extent of colonization in B6 when compared with BALB mice [23]. The HP-infected B6 mouse in this study that had an ulcer permeated with inflammatory generative cells did not exhibit a reduction in platelet count. The ELISA OD_{490} value of an HP-infected BALB mouse shown to be HP negative by PCR was 0.72; the platelet count was 96×10^4 cells/μl, and the red blood cell count was $1,163 \times 104$ cells/μl. The OD_{490} value of an HP-infected D2 mouse shown to be HP negative by PCR was 0.295; the platelet count was 128.3×10^4 cells/μl, and the red blood cell count was $1,186 \times 10^4$ cells/μl. The results of the antibody test (OD_{490}) for these mice were twice (or more than twice) the average OD value (i.e., 0.12 for noninfected BALB mice and 0.13 for noninfected D2 mice). It was thought that both mice were infected with HP by inoculation. Red blood cell counts were significantly different between HP-infected and noninfected D2 mice; however, further experiments are necessary to determine the underlying cause.

In conclusion, platelet counts in BALB mice were significantly decreased 2 months after HP infection relative to the counts in noninfected mice. This was independent of the anti-HP antibody titer and H-2 type. Thus, BALB mice infected with HP can serve as a model to investigate the mechanistic basis for the effectiveness of HP eradication therapy for ITP treatment.

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