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A clinical study on Kami-kihi-to treatment for idiopathic thrombocytopenic purpura

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Abstract

We investigated the effects of a Japanese version of a traditional Chinese medicine, Kami-kihi-to (加味帰脾湯) on platelet-associated IgG (PAIgG), platelet-binding IgG (PBIgG), and circulating immune complexes (CIC) in patients with idiopathic thrombocytopenic purpura (ITP). We also analyzed human leukocyte antigen (HLA) genotypes in Kami-kihi-to-responsive ITP patients.

1) Kami-kihi-to appears to promote the suppression of autoantibodies against platelets in chronic ITP. The effect of Kami-kihi-to of increasing the platelet count in ITP patients could be considered as an immunosuppressive mechanism.

2) CIC were detected in 16 ITP patients before Kami-kihi-to treatment, and after treatment the CIC level was significantly reduced. We hypothesize that the effect of Kami-kihi-to on PBIgG may be largely responsible for the improvement reduction in CIC, since PBIgG may be a more major component of CIC than PAIgG. Thus, the improvement reduction in PBIgG produced by Kami-kihi-to may benefit some ITP patients.

3) Kami-kihi-to did not induce an increase in FcγR expression in U937 cells. Therefore, it is thought that Kami-kihi-to leads to a decrease in PBIgG by another mechanism.

4) HLA-DR positivity was significantly increased in Kami-kihi-to-nonresponsive patients (nonresponders 30.7±4.7 %) as compared with responsive patients (responders 21.2±2.2 %) ($p<0.05$). HLA-A2 and Cw1 antigens were significantly increased in responders as compared with nonresponders. The DRB1*0901 and DPB1*0501 HLA class II alleles were also significantly increased in responders as compared with nonresponders.

We conclude that Kami-iihi-to is a useful drug in the management of ITP. However, further investigations are needed to elucidate the mechanism of action of Kami-kihi-to in ITP.

Key words idiopathic thrombocytopenic purpura, Kami-kihi-to, platelet-binding immunoglobulin G, FcγII receptor, human leukocyte antigen.

Abbreviations CIC, circulating immune complexes ; HLA, human leukocyte antigen ; ITP, idiopathic thrombocytopenic purpura ; PAIgG, platelet-associated IgG ; PBIgG, platelet-binding IgG ; PCR-RFLP, polymerase chain reaction-restriction fragment length protein.

1. Introduction

Idiopathic thrombocytopenic purpura (ITP) is a disease caused by circulating autoantibodies that react with the platelet membrane.[1,2] It is thought that platelet-associated IgG (PAIgG) is an important factor in the mechanism responsible for ITP, since increases in PAIgG are closely related to reduced platelet count in this disease.[1-4] The etiology of ITP remains unclear, but both genetic and environmental factors seem to be involved. Serological studies have long shown that there is an association between certain human leukocyte antigens and many autoimmune

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diseases. With the development of the polymerase chain reaction (PCR) technique, the identification of human leukocyte antigen (HLA) alleles at the DNA level has become possible and this has allowed more precise determination of the relationship of certain antigens to autoimmune disease.[5,6] Regarding the genetic aspects of ITP, the HLA haplotype is regarded as a potentially important factor, although its role remains unclear.

Although various methods have been used for the treatment of chronic ITP,[7-10] splenectomy and administration of corticosteroids are still the mainstays of therapy.[1,2] However, since ITP is an autoimmune disease, immunosuppression should, in theory, also be a successful method of treatment. It has recently been reported that some traditional oriental (Kampo) medicines affect hematopoiesis and immune system functioning.[11-13] In the present study, we have investigated the effects of a Japanese version of a traditional Chinese medicine, Kami-kihi-to (加味帰脾湯), on PAIgG, platelet-binding IgG (PBIgG), and circulating immune complexes (CIC) in patients with ITP. We have also analyzed HLA genotypes in Kami-kihi-to-responsive ITP patients.

2. Effects of Kami-kihi-to on autoantibodies in ITP

Kami-kihi-to (Jia-Wei-Gui-Pi-Tang ; TJ-137, Tsumura & Co., Japan) is a Chinese medicinal recipe which contains fourteen species of herbs (Table I). Kami-kihi-to was given to ten patients (two males and eight females, ranging in age from 29 to 69 years ; 48.9±13.9) with chronic ITP by oral administration for 12 weeks. Platelet count before treatment in these patients ranged 6 to $72\times10^3/\mu l$ ($33\pm20\times10^3$, mean± S.D.). None of these patients received given blood transfusions. The dose of Kami-kihi-to was 7.5 g, which is the standard daily dose for an adult. In addition, four patients received predonisolone, and two received ascorbic acid. The molecular weights of the plasma autoantibodies' target antigens were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Determination of PAIgG levels was perfomed using ELISA.

Table I The composition of Kami-kihi-to. 7.5 g of this recipe contains 5.0 g of dried extract obtained from mixed raw herbs in the following ratio.

Herb name	ratio
Astragalus root	3.0 g
Bupleurum root	3.0 g
Atractylodes lancea rhizome	3.0 g
Ginseng root	3.0 g
Hoelen	3.0 g
Polygada root	2.0 g
Gardenia fruit	2.0 g
Jujube fruit	2.0 g
Japan angelica root	2.0 g
Glycyrrhiza root	1.0 g
Ginger rhizome	1.0 g
Saussurea root	1.0 g
Zizyphus seed	3.0 g
Longan fruit	3.0 g

The platelet count increased in 7 patients, and the mean platelet count was increased from $33\pm20\times10^3/\mu l$ (mean±S.D.) to $662\pm35\times10^3/\mu l$ following Kami-kihi-to administration ($p<0.05$). The PAIgG level decreased in 8 patients (Fig. 1), and the mean PAIgG level decreased from 433.9±205.3 ng/10^7 platelets to 192.8±171.2 ng/10^7 platelets ($p<0.05$). Western blotting before and after administration revealed that in one patient, the IgG-band of molecular weight 30 kDa had disappeared after treatment (Fig. 2).

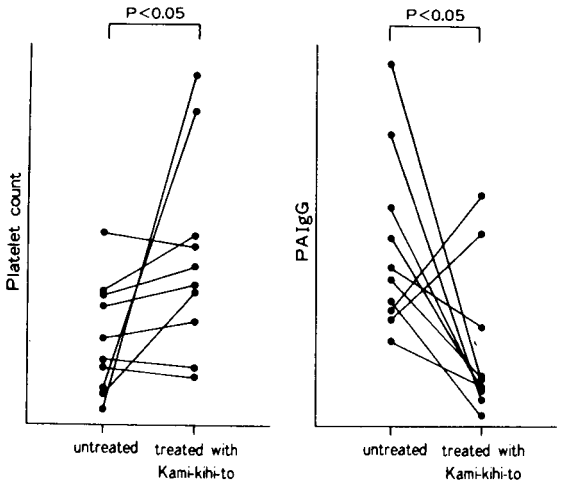

Fig. 1 Platelet count (left) and PAIgG level (right) in each patient with chronic ITP before and after administration of Kami-kihi-to.

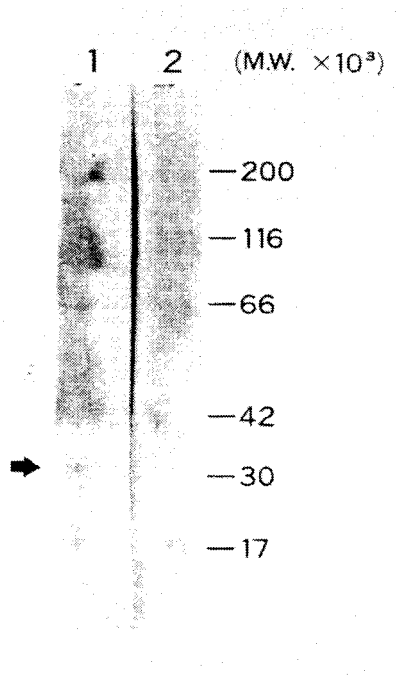

Fig. 2 Western blotting pattern of plasma of one patient for reaction with control platelet antigens. Lane 1 : before administration ; lane 2 : after administration. The numbers on the right represent to molecular weight standards. The arrow indicates a band which disappeared after administration of Kami-kihi-to.

In the present study, platelet count increased in seven out of ten patients. The disappearance of the 30 kDa IgG-band was observed in only one patient treated with Kami-kihi-to ; the PAIgG level of that patient decreased from 530 to 74 ng/10^7 platelets. Thus, Kami-kihi-to appears to promote the suppression of autoantibodies against platelets in chronic ITP. The effect of Kami-kihi-to of increasing the platelet count in these patients could be considered as an immunosuppressive mechanism.[14]

3. Effects of Kami-kihi-to on PBIgG level in ITP

We examined plasma or platelet samples from 20 patients with chronic ITP and from 15 healthy volunteers.[15] Kami-kihi-to was administered at a daily dose of 7.5 g ; patients already receiving other drugs were treated with Kami-kihi-to in addition to their standard medication. Only patients treated with the test drug for at least 3 months were evaluated. None of the subjects received any transfusions. PBIgG levels were measured by indirect immunofluorescence assay, using platelets from a selected group of type O blood donors. The samples were processed on a FACScan (Becton Dickinson & Co., CA, USA). Details of the operating conditions for FACS analysis have been published previously.[16 18] CIC level was determined by micro enzymelinked immunosorbent assay of C1q. The ELISA for C1q was performed according to the method of Kurata *et al.*[19] with some modifications. Results were expressed in terms of the percent change in alkaline phosphatase activity above or below the control level.[15]

As shown in Table II, PAIgG and PBIgG levels decreased significantly following Kami-kihi-to treatment, but the increase in the platelet count was not significant. Changes in CIC level are shown in Fig. 3. The mean±S.D. for the healthy control subjects was -0.5±7.3 %. CIC were detected in 16 ITP patients before Kami-kihi-to treatment, and the CIC level was significantly reduced after treatment ($p<0.05$). Fig. 4 shows the relationship between platelet count and antiplatelet antibodies before Kami-kihi-to administration. There was a negative correlation between

Table II Changes in platelet count, PAIgG, and PBIgG after Kami-kihi-to treatment.

	Before Kami-kihi-to	After Kami-kihi-to (12 week)	p value
Platelet count ($\times10^4/\mu l$)	3.4±2.5	4.8±3.8	N.S.
PAIgG (ng/10^7 plt)[a]	354±194	219±199	$p<0.05$
PBIgG (%)[b]	37.6±7.9	19.1±6.4	$p<0.01$

Only patients treated with Kami-kihi-to for at least 3 months were evaluated.
N.S. : not significant a : normal range (9-25) b : normal range (<10) Results are shown as the mean±S.D.
Statistical analysis of the data was performed using Studen's t-test.

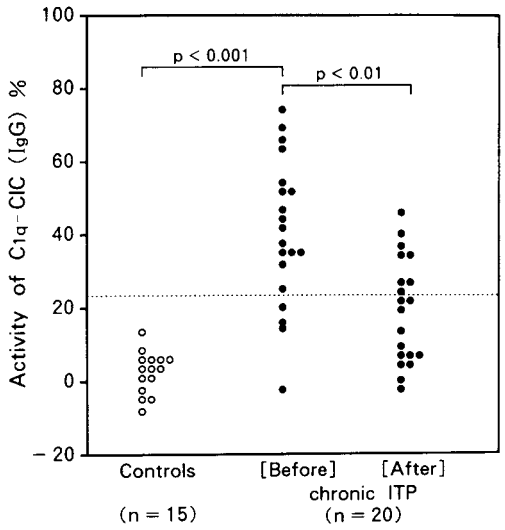

Fig. 3 Levels of CIC in patients with chronic ITP. "Before" and "After" indicate before and after (12 weeks) Kami-kihi-to administration.

platelet count and PAIgG level, but not between platelet count and PBIgG level. Figure 5 shows the changes in the PBIgG level in one patient ; the PBIgG level was reduced following Kami-kihi-to treatment.

PAIgG and PBIgG decreased significantly following Kami-kihi-to treatment, and PAIgG level was inversely correlated with platelet count. PAIgG appears to include all CIC other than specific antiplatelet antibodies, therefore a reduction in CIC may well be related to a reduction in PAIgG. It has been previously reported that most ITP sera have high levels of CIC,[19 22] and our results were similar. The role of PBIgG, on the other hand, still remains obscure. This class of IgG has been variously suggested to consist of secondary antibodies derived from platelet destruction antibodies with a low affinity for platelets, or CIC which do not play an important role

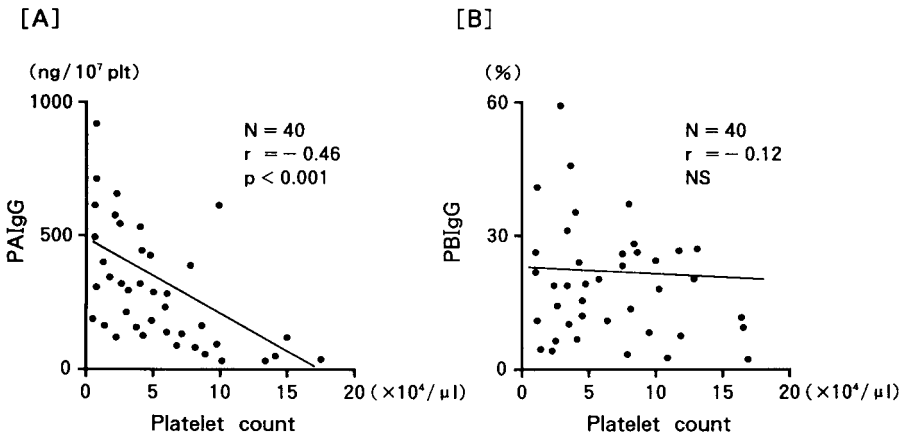

Fig. 4 Relationship between platelet count and antiplatelet antibodies (PAIgG and PBIgG) before the administration of Kami-kihi-to. There is a significant negative correlation between platelet count and PAIgG level. Statistical analysis of the data was performed using Student's *t*-test.

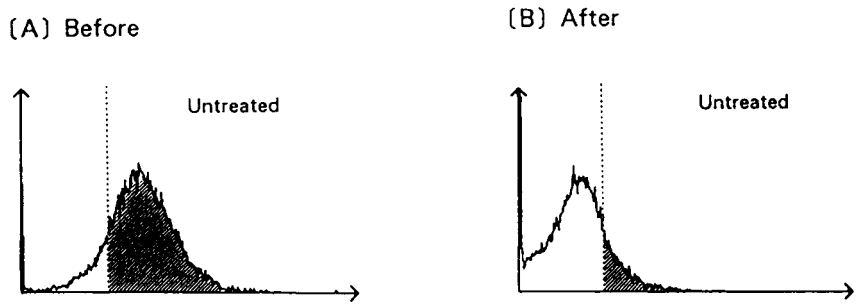

Fig. 5 Changes of PBIgG levels in ITP patient. Parafolmaldehyde-treated platelets were incubated with ITP plasma and PBIgG was detected by flow cytometry. "Before" and "After" indicate before and after Kami-kihi-to administration.

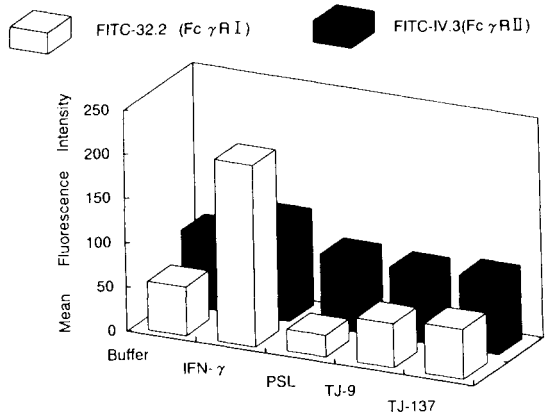

Fig. 6 Effects of drugs in binding of anti-Fcγ receptor antibodied for U937. Buffer, PBS 1 ml ; IFN-γ, 500 U ; PSL, 200 μg/ml ; TJ-9, 500 μg/ml ; TJ-137, 500 μg/ml. IFN-γ : interferon-gamma, PSL : predonisolone, TJ-9 : Sho-saiko-to, TJ-137 : Kami-kihi-to

in vivo.[1,2] Both PAIgG and PBIgG levels were reduced by Kami-kihi-to administration, and the change in PBIgG was particularly marked. We hypothesize that the effect of Kami-kihi-to on PBIgG may have been largely responsible for the improvement in CIC, since PBIgG may be more major component of CIC than PAIgG. Thus, the reduction in PBIgG induced by Kami-kihi-to may benefit some ITP patients.

4. Effect of Sho-saiko-to and Kami-kihi-to on macrophages and monocytes

We examined plasma samples from 43 patients with chronic ITP (18 untreated, 12 prednisolone-treated, and 13 Sho-saiko-to (小柴胡湯)-treated) and 15 healthy volunteers.[22] PBIgG level was measured by indirect immunofluorescence test using flow cytometry. Histocytic U937 cell lines were used to determine the effect of Kampo drugs on macrophages and monocytes. U937 cells were obtained from the American Type Culture Collection. Cells were grown in RPMI1640 medium supplemented with 10 % heat-inactivated fetal calf serum at 37°C in 5 % CO_2 atmosphere. FITC-conjugated anti-Fcγ receptor antibodies were used for this study : anti-FcγRI (FITC-32.2) and anti-FcγRII (FITC-IV.3). Light scattering technique was used to measure particle size, and FITC fluorescence was measured using a Becton Dickinson FACScan.

Untreated cases had the highest percentage of PBIgG (61.3±12.3 %). However, all treated cases showed a significant low percentage of PBIgG : corticosteroid treatment, 39.1 ± 3.4 %, $p < 0.01$; Sho-saiko-to treatment, 42.3±9.6 %, $p<0.05$; and Kami-kihi-to treatment, 40.0±10.5 %, $p<0.05$. Fig. 5 shows effect of the drug treatments on the binding of anti-Fcγ receptor antibodies to U937 cells. The binding of FITC-32.2 to U937 cells was significantly increased by γ-interferon treatment, but inversely decreased by corticosteroid treatment. The binding of FITC-IV.3 to U937 cells was slightly increased by γ-interferon treatment. The binding of both FITC-32.2 and FITC-IV.3 to U937 cells was not changed by Sho-saiko-to or Kami-kihi-to treatment.

In ITP, the recognition and binding of IgG-coated platelets by macrophage Fc-receptors leads to the sequestration of opsonized platelets by the reticuloendothelial system.[23] PAIgG, including CIC, binds to platelets via the Fc receptors on the platelet membrane. It has been reported that increased FcγR expression on the platelet membrane promotes the clearance of IgG-containing CIC from the circulation and also contributes to the development of immune complex-mediated thrombocytopenia.[24] However, Kami-kihi-to did not induce increased FcγR expression in U937 cells. This result suggests that Kami-kihi-to leads to the decrease in PBIgG by another mechanism.

5. Analysis of HLA genotypes in Kami-kihi-to-responsive ITP patients

Kami-kihi-to was orally administered to 28 patients with chronic ITP for 6 months. To make characterize Kami-kihi-to-responsive ITP patients, we measured platelet count, PAIgG, serum-IgG, serum-IgA, serum-IgM, complement $(C)_3$, C_4, LDH, and lymphocyte surface markers (CD3 · CD4 · CD8 · HLA-DR).[25] We also typed for HLA class I and HLA class II antigens. The ITP patients and the healthy controls were serologically typed for HLA class I and class II antigens using the standard complement

Table III HLA antigens and alleles in patients with ITP.

Antigen & Allele	Responder n=8	Nonresponder n=20	p value
A2	5/16 (31.3 %)	6/40 (15.0 %)	$p<0.05$
A24	3/16 (18.8 %)	15/40 (37.5 %)	N.S.
A26	3/16 (18.8 %)	6/40 (15.0 %)	N.S.
Cw1	5/16 (31.3 %)	8/40 (20.0 %)	$p<0.05$
DRB1*0901	6/16 (37.5 %)	5/40 (12.5 %)	$p<0.05$
DRB1*1502	3/16 (18.8 %)	5/40 (12.5 %)	N.S.
DQB1*0302	4/16 (25.0 %)	7/40 (17.5 %)	N.S.
DPB1*1501	6/16 (37.5 %)	9/40 (22.5 %)	$p<0.05$

Statistical analysis of the data was performed using Student's t-test.

microlymphocytotoxicity technique. Genomic DNA from patients and controls was extracted by phenol extraction of SDS-lysed and proteinase K-treated cells. The DNA amplified by the PCR-restriction fragment length protein (RFLP) method.

The response of each patient was assessed by the change in platelet count after 6 months of Kami-kihi-to therapy as follows : responders, an increase of $>2\times10^4/\mu l$ and nonresponders an increase of $<2\times10^4/\mu l$. Eight patients were responders and 20 patients were nonresponders. There were no significant differences in platelet count, PAIgG, serum-IgG, serum-IgA, serum-IgM, C_3, C_4, LDH, CD3, CD4, and CD8. However, HLA-DR positivity was significantly increased in nonresponders (30.7 ± 4.7 %) as compared with responders (21.2 ± 2.2 %) ($p<0.05$). Table III shows the HLA antigens and alleles in patients with ITP. The HLA-A2 and Cw1 antigens were significantly increased in responders as compared with nonresponders. DRB1*0901 and DPB1*0501, HLA class II alleles, were also significantly increased in responders as compared with nonresponders.

ITP is a clinically well defined autoimmune disease. As various autoimmune diseases show HLA class I and/or class II associations, several groups have tried to confirm precise relationships of HLA class I and HLA-DR antigens to ITP.[26-28] Results have been controversial, possibly because only a limited number of HLA-DR antigens have been serologically determined.[27,28] On the other hand, polymorphisms within class II genes of the major histocompatibility complex (MHC) for the HLA-DR, -DQ, and -DP antigens can now be precisely defined by typing using the PCR-RFLP method. It has been reported that aplastic anemia patients who possess HLA-DR2 are likely to respond to immunosuppressive therapy.[29,30] Thus, the analysis of HLA antigens and alleles may offer useful information regarding therapy for autoimmune diseases. Our results of HLA antigens and alleles in Kami-kihi-to-responsive ITP patients support this idea. However, most autoimmune diseases share pathogenetic mechanisms characterized by an association with an HLA class II haplotype, and the pathogenesis of ITP is clearly heterogeneous. Analysis of larger population of different genetic backgrounds will be necessary to obtain more precise information.

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