**Original** 和漢医薬学会誌 **10**, 40－46, 1993

# Effects of Kami-kihi-to on platelet-binding immunoglobulin G in idiopathic (autoimmune) thrombocytopenic purpura

Shosaku NOMURA,* Mutsumasa YANABU, Tsutomu FUKUROI, Hirofumi KIDO, Toshihiro KAWAKATSU, Kazuyuki YAMAGUCHI, Masahiko SUZUKI, Terutoshi KOKAWA and Kojiro YASUNAGA

*The First Department of Internal Medicine, Kansai Medical University*

*(Received October 19, 1992. Accepted April 19, 1993.)*

## Abstract

We investigated the effects of Kami-kihi-to (Tsumura & Co. Japan) on platelet-associated IgG, platelet-binding IgG, and circulating immune complexes in 20 patients with autoimmune thrombocytopenic purpura. There was a negative correlation ($r=-0.46$, $p<0.001$) between the platelet count and platelet-associated IgG, but not between the platelet count and platelet-binding IgG. Levels of both types of IgG, particularly platelet-binding IgG, were reduced by the administration of Kami-kihi-to. Circulating immune complexes were detected in 16 patients before Kami-kihi-to treatment, and the level of these complexes showed a significant reduction after treatment ($p<0.05$). We also measured platelet-binding IgG in the presence and absence of platelet Fc receptor blockade produced by a monoclonal anti-Fc$\gamma$ II receptor antibody (NNKY3-2), and confirmed a marked reduction of platelet-binding IgG. These results suggested that the reduction in platelet-binding IgG produced by Kami-kihi-to was due to a reduction in circulating immune complexes, with the improvement of platelet-associated IgG also being due, in part, to the same mechanism.

**Key words** Idiopathic thrombocytopenic purpura, Kami-kihi-to, platelet-binding IgG, circulating immune complex, Fc$\gamma$ II receptor.

**Abbreviations** ITP, idiopathic thrombocytopenic purpura ; PAIgG, platelet-associated IgG ; PBIgG, platelet-binding IgG ; CIC, circulating immune complex ; Kami-kihi-to, Jia-Wei-Gui-Pi-Tang.

## Introduction

Idiopathic (autoimmune) thrombocytopenic purpura (ITP) is a syndrome caused by circulating antibodies 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.[1-5] On the other hand, elevated levels of circulating immune complexes (CIC) have also been reported in patients with ITP, and the high incidence of these complexes suggests that they are also responsible for some cases of ITP.[6,7] It is thought that CIC binds to platelets via the Fc receptor and can be included in the class of PAIgG. However, very little is known regarding the immunological properties of CIC in ITP.

Although various methods have been used for the treatment of chronic ITP, splenectomy and the administration of corticosteroids are still the mainstays of therapy.[1,2] However, since ITP is an autoimmune disease, in theory, immunosuppression should also be a successful mode of treatment. It has recently been reported that some traditional oriental (Kampo) medicines affect the

*570 大阪府守口市文園町一番地
関西医科大学第一内科学教室　野村昌作
1 Fumizono, Moriguchi, Osaka 570, Japan

Journal of Medical and Pharmaceutical Society for WAKAN-YAKU **10**, 40－46, 1993

hematopoietic and immune systems.[8,10] In the present study, we investigated the effects of a Japanese version of a traditional Chinese medicine ("Kami-kihi-to") on PAIgG, platelet-binding IgG (PBIgG), and CIC in patients with ITP.

## Subjects and Methods

*Subjects* : We studied plasma or platelet samples obtained between April 1990 and August 1991 from 20 patients with chronic ITP (3 men and 17 women, aged 21-27 years) and 15 healthy volunteers. The ITP patients had thrombocytopenia, with a normal or increased number of megakaryocytes and no evidence of any underlying cause of immune platelet destruction. A profile of the ITP patients is given in Table I. Kami-kihi-to (Tsumura & Co., Japan) was given at a daily dose of 7.5 g ; patients who were 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.

*Quantitation of platelet-associated IgG (PAIgG)* : Details of the competitive solid-phase enzyme immunoassay used for the quantitation of PAIgG have been published previously.[11] The normal range of PAIgG was 9-25 ng/$10^7$ platelets.

*Measurement of platelet-binding IgG using flow cytometry* : We used the platelet suspension immunofluorescence test of Borne *et al*.[12] with flow cytometry to detect platelet-binding IgG (PBIgG).[13-15] Washed platelets were prepared as described previously.[13-15] PBIgG was measured by an indirect immunofluorescence test, using platelets from a selected group of type O blood donors. In brief, healthy washed platelets were fixed with 1% paraformaldehyde and then incubated with ITP plasma (100$\mu$l) for 30 min at room temperature. After centrifugation (1,400 g, 10 min), platelets were incubated with 5 $\mu$g/ml fluorescence-labeled goat anti-human IgG (Cappel products, PA, U.S.A.) for 30 min at room temperature, and washed 3 times by centrifugation. After a final wash, the samples were processed on a FACS analyzer (Becton Dickinson & Co., CA, U.S.A.). Details of the operating conditions for FACS analysis have been published previously.[13,14]

Table I Clinical profile of the 20 ITP patients.

| Case | Age/Sex | Duration of ITP (months) | PLT ($\times 10^4/\mu$l) | PAIgG (ng/$10^7$ plt) | PSL | $\gamma$-glob. | Spl. | Combination drug therapy (Standard medication) |
|---|---|---|---|---|---|---|---|---|
| 1 | 54/F | 19 | 5.0 | 203 | (+) | (+) | No | No |
| 2 | 50/M | 51 | 7.2 | 707 | (+) | No | No | VC (2,000 mg/day) |
| 3 | 48/F | 50 | 0.4 | 426 | (+) | (+) | No | PSL (30 mg/day), VC (2,000 mg/day) |
| 4 | 60/M | 24 | 0.4 | 528 | (+) | No | No | PSL (15 mg/day) |
| 5 | 34/F | 63 | 1.1 | 352 | (+) | (+) | (+) | PSL (10 mg/day), VC (2,000 mg/day) |
| 6 | 71/F | 24 | 5.1 | 124 | (−) | No | No | PSL (5 mg/day) |
| 7 | 56/F | 43 | 3.6 | 380 | (−) | No | No | No |
| 8 | 62/M | 54 | 3.3 | 452 | (−) | (+) | (+) | PSL (10 mg/day) |
| 9 | 27/F | 30 | 0.4 | 355 | (+) | No | No | PSL (10 mg/day), VC (2,000 mg/day) |
| 10 | 23/F | 29 | 4.2 | 249 | (+) | No | No | PSL (15 mg/day), IM (50 mg/day) |
| 11 | 41/F | 60 | 0.6 | 881 | (+) | No | No | PSL (15 mg/day) |
| 12 | 38/F | 20 | 0.7 | 263 | (+) | No | No | PSL (15 mg/day) |
| 13 | 29/F | 28 | 3.3 | 274 | (+) | No | No | No |
| 14 | 29/F | 6 | 2.5 | 254 | (+) | No | No | No |
| 15 | 69/F | 6 | 2.2 | 277 | No | No | No | No |
| 16 | 57/F | 6 | 7.7 | 53 | No | No | No | No |
| 17 | 30/F | 11 | 8.5 | 530 | No | No | No | No |
| 18 | 55/F | 69 | 1.4 | 300 | (−) | No | No | PSL (15 mg/day) |
| 19 | 65/F | 30 | 4.5 | 383 | No | No | No | No |
| 20 | 21/F | 15 | 3.6 | 113 | (+) | No | No | No |

PLT : platelet count PSL : prednisolone $\gamma$-glob.: $\gamma$-globulin Spl. : splenectomy VC : ascorbate IM : Imuran
No : not done (+) : effective (−) : ineffective

*Microenzyme-linked immunosorbent assay of C1q* : C1q was purchased from Chemicon International Inc (Tokyo, Japan). The ELISA for C1q was performed essentially by the method of Kurata *et al.*,[7] with some modifications. In brief, the wells of microtiter plates were coated by incubation overnight (at 4℃) with 0.25$\mu$g of C1q in a volume of 200$\mu$l per well. Serum obtained from patients or controls was then added and further incubation was performed. After further washing was carried out, alkaline phosphatase-conjugated anti-human IgG (Sigma, St Louis, MO, U. S.A.) was added to each well, and the plates were read with an MRP-A4 Microplate Reader (Tosoh Inc., Tokyo, Japan). Results were expressed in terms of the percent change in alkaline phosphatase activity above or below the control level, using the following formula :

$$\text{Percent change} = \frac{\text{OD Platelet extract wells} - \text{OD Control wells}}{\text{OD Control wells}} \times 100$$

Samples with a percentage increase greater than 3 SD above the mean value for the healthy controls were considered to be positive.

*Monoclonal antibody* : An anti-Fc$\gamma$ II receptor monoclonal antibody (NNKY3-2) was used. The characteristics of this antibody have been reported previously.[16]

*Statistical Methods* : Statistical analysis of the data was performed using Student's $t$-test.

## Results

As shown in Table II, PAIgG and PBIgG decreased significantly after treatment with Kami-kihi-to, but the increase in the platelet count was not significant.

Changes in the CIC level are show in Fig. 1. 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, the CIC level was significantly reduced after treatment ($p<0.05$).

Figure 2 shows the relationship between platelet count and antiplatelet antibodies before the administration of Kami-kihi-to. There was a negative correlation between the platelet count and PAIgG levels, but not between the platelet count and PBIgG levels.

Figure 3 shows PBIgG changes in a representative patient (case 17), both in the presence and absence of platelet Fc receptor blockade produced by the monoclonal anti-Fc$\gamma$ II receptor

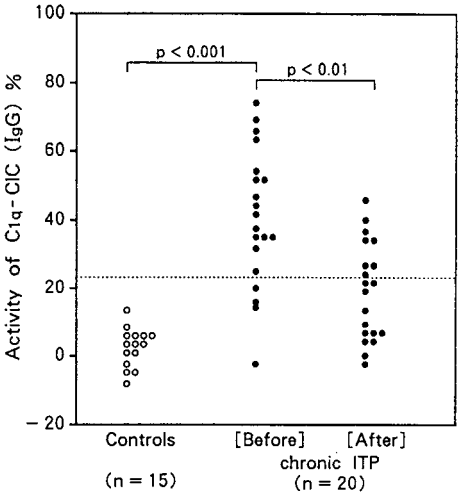


Fig. 1 Levels of circulating immune complexes (CIC) in patients with chronic ITP. "Before" and "After" indicate before and after (12 weeks) Kami-kihi-to administration.

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 |
|---|---|---|---|
| PLT ($\times 10^4/\mu$l) | 3.4±2.5 | 4.8±3.8 | NS |
| 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.
PLT : platelet count NS : not significant a : normal range (9-25)
b : normal range (<10) Results are shown as the mean±S.D.

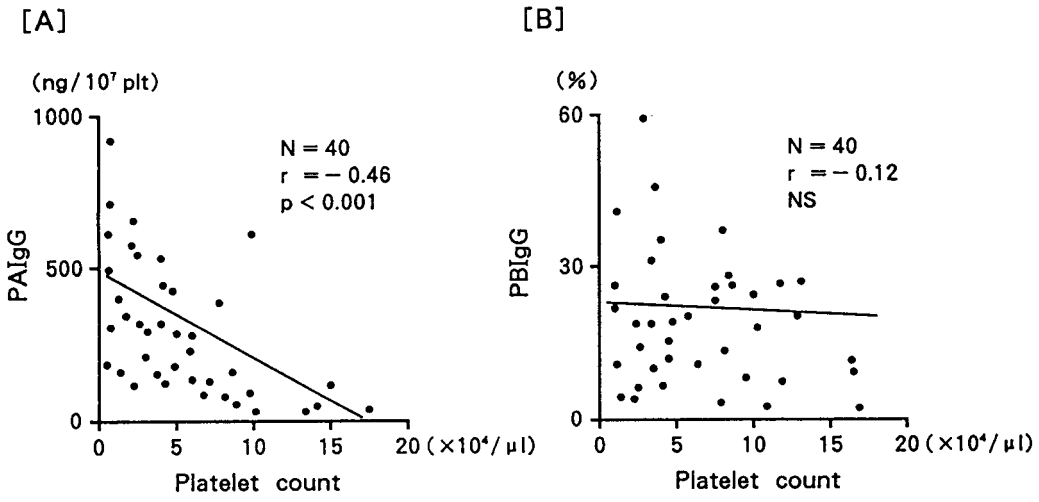


Fig. 2 Relationship between platelet count and antiplatelet antibodies (PAIgG and PBIgG) before the administration of Kami-kihi-to.
There is a significant negative correlation between platelet count and PAIgG level.

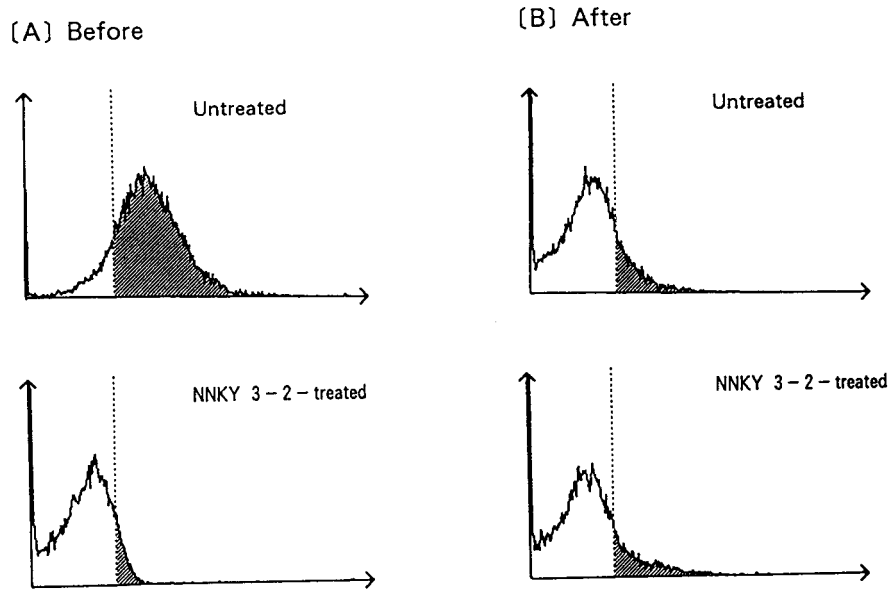


Fig. 3 PBIgG levels in case 17, with and without platelet Fc receptor blockade. PFA-treated platelets were incubated with ITP plasma and PBIgG was detected by flow cytometry. "Before" and "After" indicate before and after Kami-kihi-to administration. The upper panels show PBIgG on untreated platelets. The lower panels show PBIgG in healthy platelets after the Fc receptors were blocked with a monoclonal anti-Fcγ II receptor antibody (NNKY3-2).

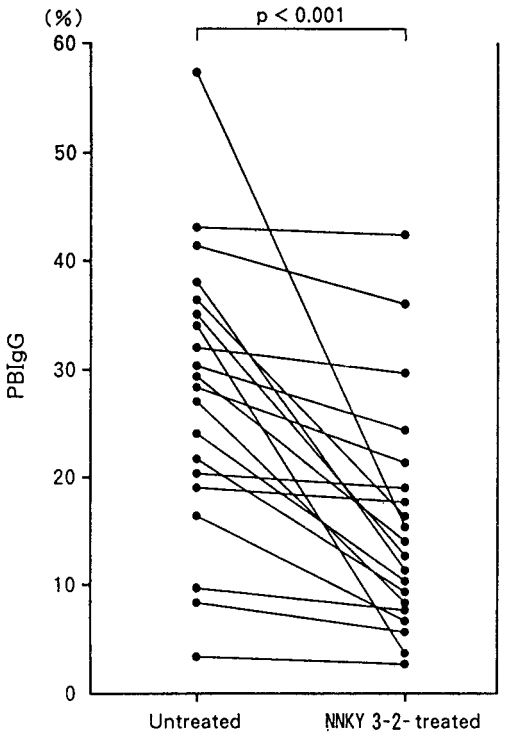


Fig. 4 PBIgG levels in all ITP patients before the administration of Kami-kihi-to, in the presence and absence of platelet Fc receptor blockade produced by NNKY3-2.

antibody (NNKY3-2). Before Kami-kihi-to treatment, the PBIgG level was markedly reduced by preincubation with the anti-Fc$\gamma$ II receptor antibody. Kami-kihi-to treatment reduced the PBIgG level compared to that before treatment, and this new post-treatment PBIgG level was little affected by preincubation with the anti-Fc$\gamma$ II receptor antibody.

Similar changes were seen in the other patients before Kami-kihi-to treatment (Fig. 4). Namely, the PBIgG levels were significantly reduced by preincubation with the anti-Fc$\gamma$ II receptor antibody ($p<0.001$).

## Discussion

This study showed that PAIgG and PBIgG were increased signficantly by treatment with Kami-kihi-to. The platelet count was also increased, but this change was not significant.

By showing that PAIgG levels were closely correlated with the severity of thrombocytopenia Borne *et al*.[12] demonstrated that PAIgG was an important factor in the mechanism responsible for ITP. In the current study, we also found that PAIgG levels were inversely correlated with the platelet count. PAIgG appears to include all CIC other than specific antiplatelet antibodies, so a reduction of CIC may well be related to a change in PAIgG. It has previously been reported that a majority of ITP sera had high levels of CIC[6,17] ; our results were similar to those reported by other authors.[6,7,17]

The significance 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 *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 hypothesized that the effect of Kami-kihi-to on PBIgG may have been largely responsible for the improvement in CIC, since PBIgG may be a larger component of CIC than PAIgG. We therefore measured PBIgG levels in the presence of the platelet Fc receptor blockade produced by a monoclonal anti-Fc$\gamma$ II receptor antibody (NNKY3-2), and we confirmed a marked reduction in PBIgG. This finding suggests that the reduction of PBIgG produced by Kami-kihi-to was related to the reduction in CIC. In general, it is thought that the specificity of antiplatelet antibody in ITP is greater in PAIgG than in PBIgG. However, it has been reported that PBIgG participates in the reduction of platelet count seen in ITP.[18] Thus, the improvement in PBIgG produced by Kami-kihi-to may benefit some ITP patients. In addition, the lesser improvement in PAIgG produced by this agent could also be related in part, to the changes in CIC, and could this lead to improvements in platelet counts in ITP.

## Acknowledgement

This work was partly supported by a Reserch Grant for Intractable Diseases from the Ministry of Health and Welfare of Japan.

## 和文抄録

特発性血小板減少性紫斑病（ITP）患者に，加味帰脾湯を投与し，投与前後の血小板表面 IgG (PAIgG)，血小板結合性 IgG(PBIgG)，および免疫複合体（CIC）を測定することによって，ITPに対する加味帰脾湯の治療効果の機序につき検討を加えた。対象は，当院で経過観察中の慢性ITP 20例である。血小板数とPAIgGの間には，負の相関関係（$r=-0.46$, $p<0.001$）がみられたが，血小板数とPBIgGとの間には相関関係はみられなかった。加味帰脾湯投与後に，PAIgG・PBIgGともに低下傾向がみられ，特にPBIgGにおいて著明であった。CICは，加味帰脾湯投与前に20例中16例において増加が検出され，加味帰脾湯投与後に有意な低下を示した（$p<0.05$）。さらに，加味帰脾湯投与前におけるPBIgGは，抗FcγIIレセプターモノクローナル抗体（NNKY3-2）で処理した血小板を用いて測定すると，有意な低下が観察された（$p<0.001$）。以上の検討より，加味帰脾湯はITP患者の血中に存在するCICを減少させることによってPAIgGやPBIgGを低下させ，抗血小板抗体による血小板減少を改善する可能性があると考えられた。

## References

1) Karpatkin, S.: Autoimmune thrombocytopenic purpura. *Blood* **56**, 329-343, 1980.
2) McMillan, R.: Chronic idiopathic thrombocytopenic purpura. *N. Engl. J. Med.* **304**, 1135-1147, 1981.
3) Dixon, R., Rosse, W. and Ebbert, L.: Quantitative determination of antibody in idiopathic thrombocytopenic purpura: correlation of serum and platelet-bound antibody with clinical response. *N. Engl. J. Med.* **292**, 230-236, 1975.
4) Arnott, J., Horsewood, P. and Kelton, J.G.: Measurement of platelet-associated IgG in animal models of immune and nonimmune thrombocytopenia. *Blood* **69**, 1294-1299, 1987.
5) Oyaizu, N., Yasumizu, R., Miyama-Inaba, M., Nomura, S., Yoshida, H., Miyawaki, S., Shibata, Y., Mitsuoka, S., Yasunaga, K., Morii, S., Good, R.A. and Ikehara, S.: (NZW×BXSB) $F_1$ mouse. A new animal model of idiopathic thrombocytopenic purpura. *J. Exp. Med.* **167**, 2017-2022, 1988.
6) Lurhuma, A.Z., Riccomi, H. and Masson, P.L.: The occurrence of circulating immune complexes and viral antigens in idiopathic thrombocytopenic purpura. *Clin. exp. Immunol.* **28**, 49-55, 1977.
7) Kurata, Y., Hayashi, S., Aochi, H., Nagamine, K., Oshida, M., Mizutani, H., Tomiyama, Y., Tsubakio, T., Yonezawa, T. and Tarui, S.: Analysis of antigen involved in circulating immune complexes in patients with idiopathic thrombocytopenic purpura. *Clin. exp. Immunol.* **67**, 293-299, 1987.
8) Kawamura, H., Maruyama, H., Takemoto, N., Komatsu, Y., Aburada, M., Ikehara, S. and Hosoya, E.: Accerating effect of Japanese kampo medicine on recovery of murine hameatopoietic stem cells after administration of mitomycin C. *Int. J. Immunotherapy* **5**, 35-42, 1989.
9) Satomi, N., Sakurai, A., Imura, F., Haranaka, R. and Haranaka, K.: Japanese modified traditional Chinese medicines as preventive drugs of the side effects induced by tumor necrosis factor and lipopolysaccharide. *Mol. Biother.* **1**, 155-162, 1989.
10) Ohnishi, Y., Yasumizu, R., Fan, H., Liu, J., Takao-Liu, F., Komatsu, Y., E., Good, R.A. and Ikehara, S.: Effects of Juzen-taiho-toh (TJ-48), a traditional oriental medicine, on hematopoietic recovery from radiation injury in mice. *Exp. Hematol.* **18**, 18-22, 1990.
11) Tsubakio, T., Kurata, Y., Yonezawa, T. and Kitani, T.: Quantification of platelet-associated IgG with competitive solid-phase enzyme immunoassay. *Acta Haematol.* **66**, 251-256, 1981.
12) Borne, A.E.G.Kr. von dem, Hermerhorst, F.M., van Leeuven, E.F., Pegels, H.G., von Riesz, E. and Engelfriet, C.P.: Autoimmune thrombocytopenia - Detection of platelet autoantibodies with the suspension immunofluorescence test. *Br. J. Haematol.* **45**, 319-327, 1980.
13) Nomura, S., Nagata, H., Oda, K., Kokawa, T. and Yasunaga, K.: Effects of EDTA on the membrane glycoprotein IIb-IIIa complex - analysis using flow cytometry. *Thromb. Res.*, **47**, 47-58, 1987.
14) Nomura, S., and Kokawa, T.: Recent advance on analysis of antiplatelet antibody - special reference to application of flow cytometry. In *"Automation and New Technology in the Clinical Laboratory"* (Ed. by K. Okuda), Blackwell Scientific Publications, Oxford, pp. 287-294, 1990.
15) Nomura, S., Yanabu, M., Kido, H., Fukuroi, T., Yamaguchi, K., Soga, T., Nagata, H., Kokawa, T. and Yasunaga, K.: Antiplatelet autoantibody-related microparticles in patients with idiopathic (autoimmune) thrombocytopenic purpura. *Ann. Hematol.* **62**,

103-107, 1991.

16) Nomura, S., Yamaguchi, K., Kido, H., Kawakatsu, T., Iwata, K., Fukuroi, T., Suzuki, M., Yanabu, M., Soga, T., Nagata, H., Kokawa, T. and Yasunaga, K. : New monoclonal anti-human Fc gamma receptor II antibodies induce platelet aggregation. *Clin. exp. Immunol.* **86**, 179-184, 1991.

17) Trent, R.J., Clancy, R.L., Danis, V. and Basten, A. : Immune complexes in thrmbocytopenic patients : cause or effect ? *Br. J. Haematol.* **44**, 645-654, 1980.

18) Mizumoto, Y., Nishikawa, K., Fujimura, Y., Morii, T., Narita, N., Fukui, H. and Kurata, Y. : Acid treatment of platelets increased the specificity for the detection of platelet binding IgG in patients with idiopatic thrombocytopenic purpura by flow cytometry. *Kessen-Shiketsu-shi* **1**, 84-93, 1990 (in Japanese).