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Studies on Administration of Aspirin in Thrombocytosis: Individualization of Minimum Dose Administration and Effect of Thromboxane Synthetase Inhibitor on Spontaneous Platelet Aggregation *in vitro*

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Summary. **This study aims to determine the minimum doses of aspirin required to inhibit spontaneous platelet aggregation (SPA) in patients with thrombocytosis. *In vitro* effects of a thromboxane synthetase inhibitor (OKY-1581) and aspirin on SPA were examined. Aspirin *in vitro* inhibited SPA at 10^{-4} M whereas OKY-1581 did in wide range of 36×10^{-7} - 36×10^{-4} M. In an *in vivo* assessment of aspirin, nine patients who showed SPA were tested for such as SPA, aggregation induced by ADP, collagen, arachidonate and malondialdehide production after daily administration of 10-60 mg of aspirin for at least one week. The aspirin inhibited SPA in a dose dependent manner. Although varing among patients, the minimum doses of aspirin, ranging from 10 to 60 mg, were far lower than the doses described thus far in the literature (500-1,000 mg), and changed during the observation period. For patients with thrombocytosis accompanying arterial insufficiency, the dosage of aspirin should be determined individually based on assessments regarding platelet aggregation.**

INTRODUCTION

The term "Aspirin Dilemma"[1] expresses a difficulty in obtaining an inhibition of platelet cyclooxygenase without an inhibition of vascular cyclooxygenase[2-4] when aspirin is administered as an antithrombotic drug. To avoid aspirin dilemma, small doses of aspirin, i.e., a daily administration of 10-80 mg, have been proposed or examined.[5-7] These proposals are based on the facts that platelet cyclooxygenase is more sensitive to aspirin than vascular cyclooxygenase, that the inhibition is irreversible in the former whereas it is reversible in the latter,[8] and that the effect of aspirin is cumulative and dose dependent.[9] Aspirin affects platelet cyclooxygenase before affecting general circulation by preserving general cyclooxygenase in vessels,[10,11] though inhibition of vascular thromboxane A_2 without influencing prostaglandin I_2 seems impossible.[12] Other factors that modulate the effect in man are such as sex,[5] salicylate,[1,13] and drug form.[14]

In primary thrombocythemia, arterial insufficiency and/or thrombosis, which are frequently fatal complications,[15] can be prevented by daily administration of aspirin (500-1,000 mg).[16-20] Since aspirin's effect is well demonstrated on the disappearance of spontaneous platelet aggregation (SPA) using conventional aggregometers,[14,18] SPA has been employed as a parameter for thrombotic tendency. We claimed administration of 500 mg once every two or three days,[21] however, it was obviously impossible to avoid the risk of hemorrhage and thrombosis even with this dose of aspirin. Thrombotic tendency in thrombocythemia could be enhanced by the inhibition of vessel cyclooxygenase, which results in inhibition of prostaglandin I_2.[5]

This study was undertaken to find the minimum dose of aspirin to inhibit SPA for at least one week of daily aspirin administration. *In vitro* effects of aspirin and a thromboxane synthetase inhibitor (OKY-1581) on SPA were examined to elucidate the role of thromboxane A_2.

PATIENTS

The subjects were seven patients with primary

Table 1. Patient profiles

	Patient		Diagnosis	Thrombohemorrhage	Plt	Rbc	Hb	Wbc	Hepato(H)-spleno(S)megaly
1	HK	71, F*	Polycythemia vera	None	1070	5.5	12.4	12(1)	H, S
2	KH	37, M	Primary thrombocythemia	None	2100	4.5	13.9	11(0)	(−)
3	HS	66, F	Primary thrombocythemia	Headache	1400	4.2	12.5	8.6(0)	(−)
4	FW	72, F	Primary thrombocythemia	TIA	2410	4.1	11.4	10(0)	(−)
5	FI	64, M	Primary thrombocythemia	None	2560	3.3	11.8	8.4(0)	(−)
6	FsW	54, F	Chronic myelo-proliferative syndrome	Splenic infarction Pulmonary infarction	750	3.1	7.6	9.3(1)	H
7	SK	76, F	Primary thrombocythemia	Vein thrombosis	840	3.6	10.1	14(0)	(−)
8	IT	53, F	Primary thrombocythemia	Acrocyanosis	2190	4.2	13.2	7(0)	(−)
9	TI	69, F	Primary thrombocythemia	Cerebral infarction	1530	5.3	12.9	13(0)	(−)

Plt: Platelets 10^9/l, Rbc: Red blood cells 10^{12}/l, Hb: hemoglobin g/dl, Wbc: White blood cells 10^9/l, ( ): % leukocytes younger than myleocytes, * age, sex The blood cell counts represent those at the start of this study.

thrombocythemia, one with polycythemia vera, and one with a chronic myeloproliferative disorder (2 males and 7 females) who all had SPA in platelet aggregometry. Clinical and laboratory data are shown in Table 1. Platelet counts ranged from 750 to 2,560 ($\times 10^9$/L). During this study the patients had neither thrombotic nor hemorrhagic symptoms, except for one (Case 8, who showed arterial insufficiency [acrocyanosis] in her fingers), and they had not taken any drugs that might affect platelet function or production.

MATERIALS AND METHODS

Aspirin administration: Twenty mg of aspirin was initially administered orally once a day for one or two weeks in 5 patients. Then the dosage was generally increased to 30, 40 and 50 mg per day over a period of at least one week. Two patients started with 30 mg per day, and one started with 50mg per day. Sixty or 100 mg per day was given in two patients. In one of the other two, whose SPA became negative by the administration of 20 mg per day, the dose was reduced to 10 mg per day. Blood was collected before and on the final day of administration. In three patients, dose dependency was examined two or three times after more than two-month intervals between examinations. Thirteen trials were performed in total in 9 patients.

Platelet function: Platelet rich plasma (PRP) was obtained by centrifugation (120G, 10 min) from citrated whole blood. SPA was observed on this original(o)-PRP (platelet count ranged 1,240-4,500$\times 10^9$/L). Collagen- (Horm, CA, USA; 2 μg/ml at final concentration), ADP- (Sigma, MO, USA; 10 μM), or arachidonate (Sigma, MO, USA; 2 mM)-induced aggregation was measured using diluted(d)-PRP (platelet count 300×10^9/L) in a conventional aggregometer, Hematracer-1 (Niko, Tokyo, Japan). In some patients aggregation was further induced by adrenalin (Daiichi, Japan; 5 μM), STA_2, a thromboxane analog (Ono, Osaka, Japan; 2 μM), or A23187 (Calbiochem. CA, USA; 5 μM). Two hundred μl of PRP in a cuvette was stirred by a steel stirrer (1 mm$\times$5 mm) at 1,000 rpm. SPA was judged as positive when the maximum aggregation ratio (MAR) exceeded 10% during a 12 min stirring without an agonist. Aggregability induced by the agonists was expressed by the maximum aggregation ratio of less than 5 min in cases of ADP-, arachidonate-, or STA_2-induction, and less than 8 min in case of collagen- or A23187-induction.

Malondialdehide measurements: malondialdehide production was measured on washed platelets activated by arachidonate 0.2 mM for 2 min by the method reported by Takayama et al.,[22] as an indicator of platelet cyclooxygenase activity in order to examine the pharmachological effect of aspirin. The normal range was 1.55 ± 0.39 nM/10^8 platelets (plts) (mean$\pm$SD).

The effect of each of aspirin and OKY-1581 on SPA: SPA was examined on o-PRP that had been incubated for 30 min at 20-25°C with various concentration of aspirin (Sigma, MO, USA; up to 10^{-3} M at final concentrations) or OKY-1581 (Ono, Osaka, Japan; up to 1 mg/ml, 36×10^{-4} M at f.c.), a thromboxane synthetase inhibitor, or with a vehicle.

Statistical analysis: The data were compared by using paired or non-paired t-test, F-test, Aspin-Welch,

and/or Cochran-Cox methods. P of less than 0.05 was considered significant.

RESULTS

1. Pre-aspirin administration findings

SPA was found in all the patients; maximum aggregation ratios ranged between 45 and 90%. The lag times ranged from 1.5 to 11.0 min. Malondialdehide amounts ranged from 0.69 to 2.05 nM/10^8 plts, except for a slightly lower value in one patient. Aggregability induced by agonists using d-PRP was within normal range (data not shown) in most patients and was slightly lower in a few patients.

2. Effects of aspirin administration

SPA (Table 2): Daily administration of 10 mg of aspirin for at least a week suppressed SPA in 1 out of 6 trials (1/6), and similarly 20 mg of aspirin suppressed SPA in 6/9, 30 mg of aspirin in 8/11, 40 mg in 8/10, 50 mg in 5/7, 60 mg in 2/2, and 100 mg (data not shown) in 1/1. In one patient SPA was positive once after an administration of 30 mg but was negative after a second administration of the same dose. However, SPA was negative after both the first and second administrations at 40 mg, suggesting that this was the minimum dose. The minimum dose estimated as above was found to be 10 mg in 1 trial, and similarly 20 mg in 5 (including a possible 1), 30 mg in 2, 40 mg in 2 and 50 mg in 1, and 60 mg in 2. Thus the effect of aspirin considerably differed among the patients, though it was generally dose dependent. Acrocyanosis in case 8 (IT) almost disappeared when 40 mg of aspirin was given and coincided with the disappearance of SPA.

Agonist-induced aggregation: Aspirin at more than 20 mg per day showed significant inhibition of aggregability of d-PRP induced by collagen. At a dose of 50 mg per day, aspirin almost abolished collagen-induced aggregation. A significant inhibition was also seen with arachidonate (except 30 mg per day and 40 mg per day). On the contrary, the aggregability with ADP 10 μM was not affected even at 50 mg per day, as shown in Table 3. When the aggregability during this trial was compared between SPA-positive and negative samples regardless of the dose of aspirin, SPA-positive samples showed higher aggregability with either collagen or arachidonate (using non-paired t-test, Aspin-Welch, F-test and Cochran-Cox methods) but they showed similar aggregability in the case of induction with ADP 1 μM and 10 μM, adrenalin, or A23187 5 μM (Table 4). A close relationship was found between SPA (maximum aggregation

Table 2. Individualization of Aspirin Mimimum Dose in Primary Thrombocythemia
Parameter: Spontaneous platelet aggregation (SPA)

Trial	Patient	Aspirin administration (mg/day)#							Minimum dose	Date
		0	10	20	30	40	50	60		
1	HK 71 F	80*	66	-**	-				20	Jan. 1989
2		75	-	-	-				10	Mar. 1989
3	KH 37 M	65	80	-	-	-			20	Dec. 1987
4		67	70	10	-				30	Nov. 1988
5	HS 66 F	80		-	-	-			20(≧)	Oct. 1983
6		75				70	90/-	-	60	May. 1984
7		50	72	-	-	-	-		20	Feb. 1985
8	FW 72 F	60	70	-	-	-			20	1986
9	FI 64 M	74		64	70	46		-	60(≧)	Aug. 1984
10	FsW 54 F	45		51		-	-		40(≧)	Aug. 1984
11	SK 76 F	78			-	-			30(≧)	Aug. 1984
12	IT 53 F	65			62	-	-		40	Apr. 1985
13	TI 69 F	65			72	-	40/-		(≧)50	Sep. 1988
	SPA	13/13	5/6	3/9	3/11	2/10	2/7	0/2		

One a day, for at least 1 week, * Maximum aggregation rate (%), ** less than 10%

Table 3. Effects of aspirin on collagen-, arachidonate- or ADP-induced aggregation in diluted PRP.

Agonist		Aspirin (mg/day) 10	20	30	40	50
Collagen 2 μg/ml	A	72.0±0 (3)*	69.2±14.3 (21)	70.2±16.0 (17)	65.9±19.0 (10)	71.9±20.8 (10)
	B	27.7±37.0 (3)	28.1±33.8 (21)	28.5±35.5 (17)	10.0±19.1 (10)	0.4± 0.8 (10)
		NS	0.001	0.005	0.001	0.001
AA 2mM	A	74.0±0 (3)	82.2± 9.6 (21)	81.3±12.0 (16)	77.0±46.9 (9)	67.8±16.5 (12)
	B	84.0±6.5 (3)	41.2±37.1 (21)	57.1±37.8 (16)	46.9±33.3 (9)	23.9±32.9 (12)
		NS	0.001	0.1>0.05	NS	0.01
ADP 10 μM	A		58.7±34.5 (14)	61.7±31.9 (13)	67.5±19.0 (8)	66.0±28.4 (7)
	B		59.4±31.4 (14)	75.2±16.2 (13)	72.4±15.0 (8)	65.9±11.4 (7)
			NS	NS	NS	NS

A: before administration, B: after administration, * MAR (%), mean±SD mean (sample number) NS: not significant.

Table 4. Comparison of aggregability induced by agonists between positive and negative SPA during asprin trial

Agonist	ADP 1 μM	ADP 10 μM	Collagen	Arachidonate	Adrenalin	A23187 5 μM
SPA positive	17.0±18.3 (26)	72.2±22.5 (26)	52.8±35.2 (32)	62.9±34.4 (19)	25.0±38.1 (19)	50.7±34.4 (7)
negative	17.3±18.1 (35)	65.6±21.9 (34)	19.9±33.6 (38)	38.0±38.6 (45)	20.1±33.5 (26)	53.2±47.6 (9)
P value	>0.1	>0.1	<0.001	<0.005	>0.1	>0.1

Values are given as Mean±SD (sample number) of MAR (%)

Table 5. Correlation between parameters during aspirin trials

Parameters* y		x	r	(no)		p
SPA	VS	ADP 1 μM	−0.117	(57)	y=−0.055x+17.87	0.3
	VS	ADP 10 μM	0.276	(58)	y=0.239x+57.25	0.05
	VS	collagen	0.386	(75)	y=0.430x+30.69	0.001
	VS	arachidonate	0.412	(73)	y=0.458x+42.06	0.001
	VS	STA_2	−0.063	(35)	y=−0.32x+81.06	0.5
	VS	adrenalin	0.191	(43)	y=0.210x+18.64	0.3
	VS	A23187 5 μM	0.001	(15)	y=0.009x+47.66	0.4
ADP 1 μM	VS	ADP 10 μM	0.469	(65)	y=0.694x+57.74	0.001
ADP 10 μM	VS	collagen	0.141	(66)	y=0.399x+22.52	0.3
collagen	VS	arachidonate	0.485	(80)	y=0.483x+34.59	0.001

* Values expressed by MAR (% maximum aggregation)

ratio, %) and maximum aggregation ratio by collagen or arachidonate (Table 5).

Malondialdehide in relation to SPA or arachidonate-induced aggregation: Malondialdehide production decreased as the aspirin dose increased, and SPA usually became negative (Fig. 1) and arachidonate-induced aggregation was generally eliminated (Fig. 2) when the malondialdehide was below 0.4 nM/10^8 plts,

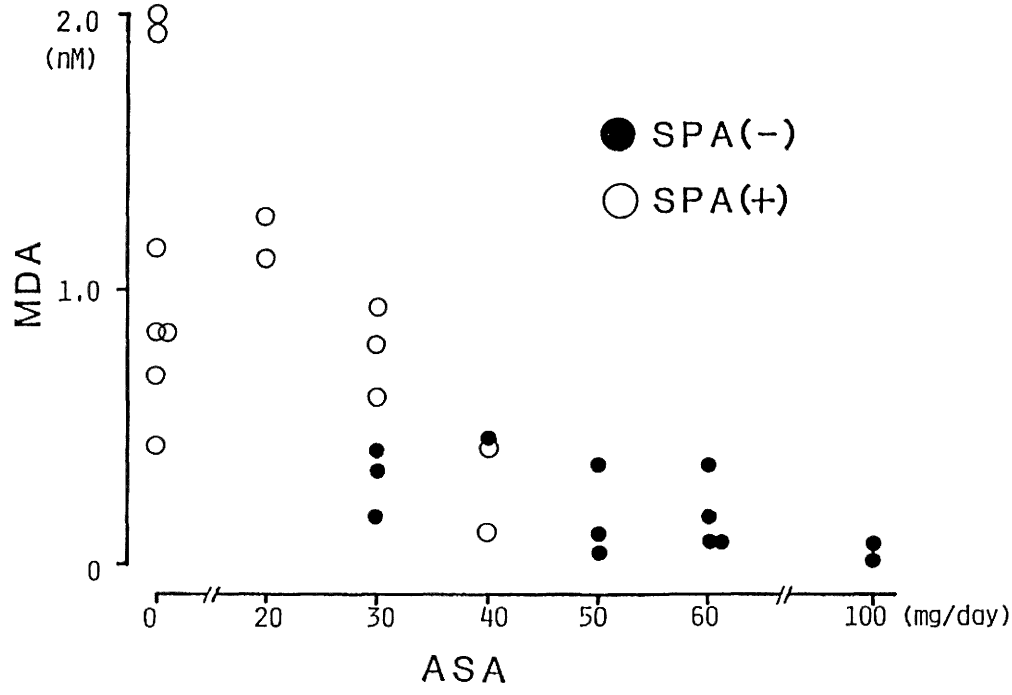

Fig. 1. Effect of ASA administration on MDA production and SPA. MDA amounts were generally suppressed as the ASA dose was increased. SPA became negative when MDA was lower than approximately 0.3 nM/10^8 plts. In two exceptional patients after administrating 40 mg of daily, SPA remained positive in spite of the lowered MDA values.

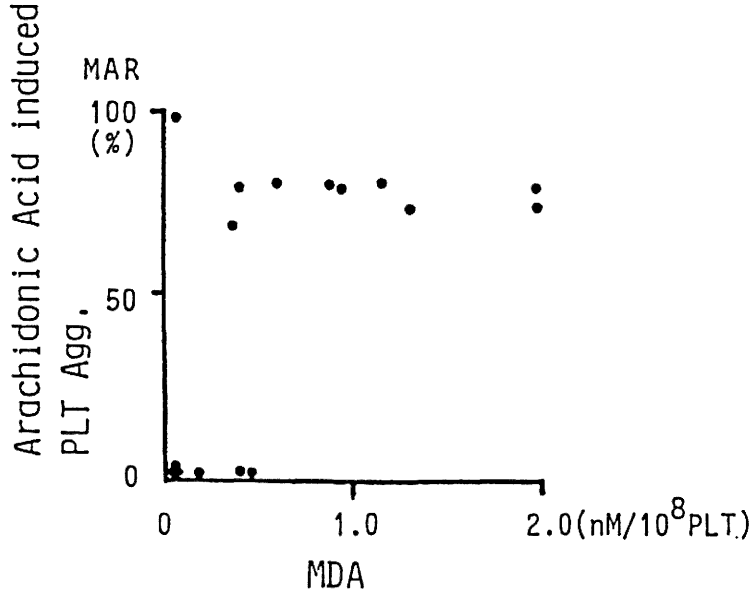

Fig. 2. Relationship between arachidonate-induced aggregation and MDA production. The points represent each result obtained before and during administration of various doses. Generally, aggregation was induced normally when MDA exceeded approximately 0.5 nM/10^8 plts, and defective when below 0.3 nM except for one case (Case 6: FsW).

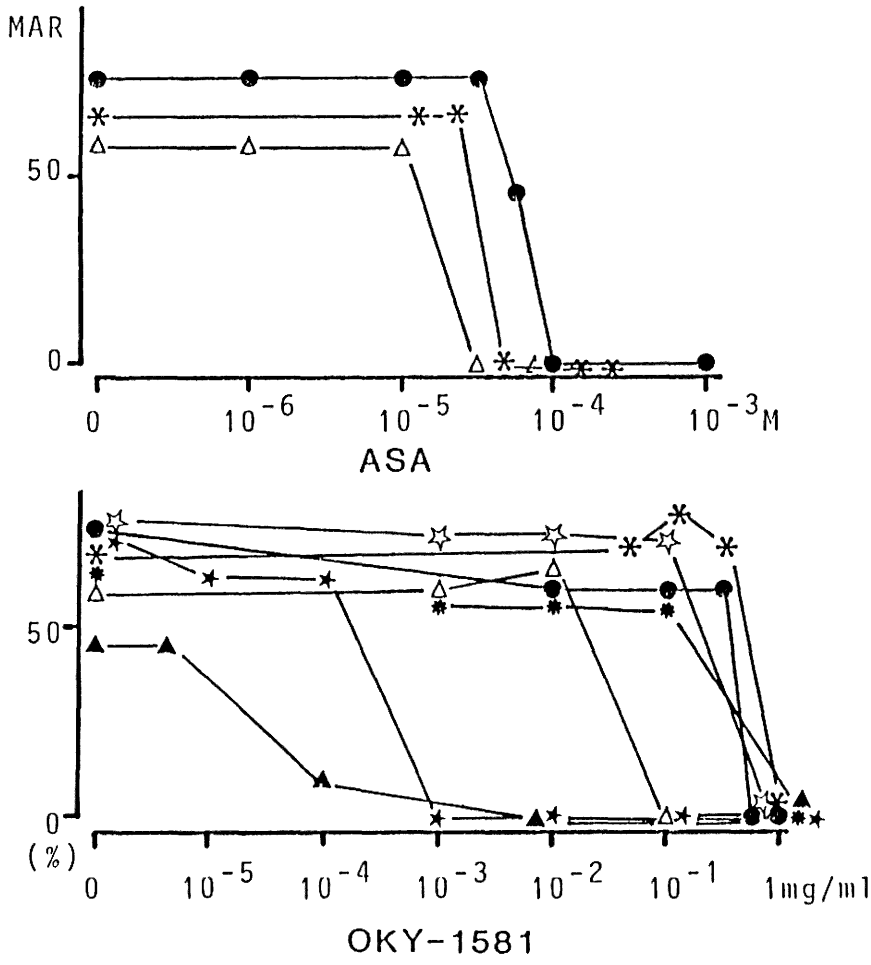

Fig. 3. Inhibition of SPA by ASA or OKY-1581 (a thromboxane synthetase inhibitor). The original platelet rich plasma was incubated with various concentrations of the reagents or vehicle, and then examined for SPA. ASA eliminated SPA at over 3×10^{-5} - 10^{-4} M. OKY could also eliminate SPA, but the minimum concentrations for that ranged widely (10^{-4} - 1 mg/ml).

suggesting that this level of malondialdehide is critical.

3. *In vitro* effect of aspirin or OKY-1581 on SPA

Aspirin eliminated SPA in 2 of 3 patients at 3×10^{-5} M and in all patients at 10^{-4} M. OKY inhibited SPA in 2 of 7 patients at around 10^{-3} mg/ml, and in 3 at 10^{-1} mg/ml, and in all patients at 1 mg/ml (36×10^{-4} M) (Fig. 3). OKY at 1 mg/ml suppressed thromboxane B_2 (measured by RIA) production of normal platelets challenged by arachidonate down to 1/40 of control (data not shown).

DISCUSSION

Hyperreactive platelet responses are associated with acute vascular events, such as cerebral ischemia and myocardial infarction.[23-25] For prevention of these events, clinical trials of aspirin therapy, in daily dosage of 1,000-1,500 mg or recently 325 mg, have been employed.[26] To avoid the inhibition of vascular cyclooxygenase (prostaglandin I_2), dosage of aspirin must be reduced. Preston et al.[6] found an almost complete and persistent inhibition of vascular cyclooxygenase in biopsied forearm veins of normal volunteers after a single administration of 300 mg of aspirin or 160 (40×4) mg of aspirin. Kallmann et al.[27]

found no decrease in the urinary excretion of 6-keto-prostaglandin F_{1a} in normal subjects undergoing daily administration of 10 mg or 30 mg of aspirin, and claimed that their results proved retention of vascular cyclooxygenase (prostaglandin I_2). Patrignani et al.[9] also reported a similar finding. However, their idea of using the urinary excretion of I_2 metabolites as proof of retained cyclooxygenase in general vessels has been discounted by subsequent studies by Jones and Russel,[28] Bucchi et al.[29] and Lee and Hsieh.[30] Since the direct measurement of lowered, as well as normal, plasma levels of 6-keto-prostaglandin F_{1a} still remains technically difficult, evaluation of vascular cyclooxygenase in patients being administered aspirin needs further study. Hence, individualization of minimum dose of aspirin can be a reasonable and practical approach to the effective use of aspirin if there are suitable parameters for such determination.

The present study individualized the minimum aspirin doses by using SPA as a parameter based on the assumption[14,18] that the disappearance of SPA is correlated with the inhibition of at least some thrombotic episodes in this type of patient. SPA[16,20,31,32] has occasionally been demonstrated in various kinds of thrombotic disorders[16,31-33] and usually in primary thrombocythemia particularly in patients who have arterial insufficiency or thrombosis.[17-21,32] SPA can be inhibited by the administration or incubation of PRP with other antiplatelet drugs, such as ticlopidine,[34] flurbiprofen,[35] bencyclane,[36] sulfinpyrazone[37] and dipyridamole.[38] From our experience, aspirin seems to be most effective for patients with primary thrombocythemia.[21,34,36]

The minimum doses are far less than the doses previously reported (500-1,000 mg).[16-20] They varied remarkably among patients and further changed somewhat during the observation period. Since the disappearance of SPA was generally accompanied by either a remarkable reduction or total loss of aggregation induced by collagen or arachidonate, and of malondialdehide production, these daily 10-60 mg of aspirin may be adequate for each patient. The clinical effect of these very small doses of aspirin was not confirmed in the patients in the present study, except for one patient who had acrocyanosis of her fingertips. Although 60 mg of aspirin was considered enough to inhibit not only SPA and malondialdehide but also arachidonate- or collagen-induced aggregation, there was one patient (Case 6: FsW) whose malondialdehide was less than 0.05 while arachidonate-induced aggregation (maximum aggregation ratio) was near 100% (Fig. 2). Svensson and Samuelsson,[39] who also noticed a similar discrepancy in some thrombotic patients, suggested that the aspirin dose should be controlled based not on the inhibition of platelet cyclooxygenase but on that of aggregation. Our exceptional patient (Case 6) after receiving 40 mg of aspirin had, however, no SPA, thus attaining the goal of this study.

This study on SPA has confirmed the effect of administered aspirin in very small doses and also showed the efficacy of OKY-1581, although it was apparently less potent. Interestingly, the effect of OKY-1581 differed remarkably among the patients, suggesting that the mechanism of SPA varied among them. Generally, responders and non-responders to thromboxane synthetase inhibitors have been known to exist.[40] Under these circumstances prostaglandin H_2 and other prostaglandin metabolites (D_2, E_2, etc.) have been speculated to play in the appearance of SPA. For patients with primary thrombocythemia, abnormalities have been reported concerning cyclooxygenase and platelet lipoxygenase activity,[21,41] and also the production, degradation,[42,43] and receptor[44] of prostaglandin D_2.[45] Our preliminary study suggested that thromboxane, in balance with prostaglandin D_2 may influence the appearance of SPA.[46] Thus the differences among the patients in the effect of OKY on SPA should be closely examined further. In any case, the attempt to produce high concentrations of OKY to obtain uniform effect would be difficult for clinical applications. Michiels et al.[47] reported that dazoxiven (1,600 mg/day), a thromboxane synthetase inhibitor, suppressed malondialdehide production but did not relieve erythromelalgia (the number of the patients and effect on SPA were not described). In our experience, administration of OKY-046 (300-600 mg, orally), a thromboxane synthetase inhibitor, to two patients was also ineffective in abolishing SPA in both patients and in relieving erythromelalgia in one of them.

In conclusion, the present study shows that individualized daily 10-60 mg doses of aspirin may be adequate for patients with thrombocytosis who show arterial insufficiency or thrombosis. Sixty mg of aspirin may be given to such patients when no time can be spared to examine the dose dependency of aspirin's effect on SPA. This study does not negate other kinds of antiplatelet therapy,[48-51] such as thromboxane receptor antagonist and selective thromboxane sythetase inhibitors, that are free from the aspirin dilemma or myelosuppression therapy or plateletapheresis[13] for patients with venous thrombosis. No therapy may be needed for young patients.[52,53] This study has also shown that OKY inhibits SPA. A

considerable difference of its inhibitory effect among patients suggests different degrees of influence of thromboxanes on the appearance of SPA. Finally, until more suitable parameters become available, the individualization of the minimum dose of aspirin may be useful also for patients with thrombosis.

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