Platelet-activating factor: mediator of the third pathway of platelet aggregation? A study in three patients with deficient platelet-activating factor synthesis.

A Sturk, … , H Przyrembel, P Borst

J Clin Invest. 1987;79(2):344-350. https://doi.org/10.1172/JCI112818.

Research Article

Thrombin, collagen, and Ca2+-ionophore A23187 aggregate platelets in the presence of inhibitors of the first (ADP-mediated) and second (cyclooxygenase-dependent) pathway of platelet activation. This aggregation, via a third pathway, was hypothesized to be mediated by the alkoxyether lipid platelet-activating factor (PAF). We recently demonstrated virtual absence of plasmalogen-type alkoxyether lipids and deficiency in key enzymes of their biosynthesis in Zellweger patients. We hypothesized that PAF synthesis might also be impaired. We report two Zellweger patients with an undetectable A23187-induced PAF synthesis of leukocytes (patients, less than 3 pmol PAF/10(8) granulocytes (PMN); four age-matched controls, 249-2,757 pmol PAF/10(8) PMN; five adult controls, 291-5,433 pmol PAF/10(8) PMN). In a third patient, residual PAF synthesis was detected. However in all patients the thrombin-induced third mechanism of platelet aggregation was present. We therefore conclude that PAF may not be the mediator of the third pathway.

Platelet-activating Factor: Mediator of the Third Pathway of Platelet Aggregation?

A Study in Three Patients with Deficient Platelet-activating Factor Synthesis

Augueste Sturk,* Marianne C. L. Schaap,* Jan Wouter ten Cate,* Hugo S. A. Heymans,‡ Ruud B. H. Schutgens,‡ Hildegard Przyrembel,§ and Piet Borst‖

**Division of Hemostasis and Thrombosis, Department of Hematology, and ‡Department of Pediatrics, University Hospital, Academic Medical Center, Amsterdam; §Department of Pediatrics, Sophia Kinderziekenhuis, Rotterdam; and ‖Antoni van Leeuwenhoekziekenhuis, The Netherlands Cancer Institute, Amsterdam, The Netherlands*

Abstract

Thrombin, collagen, and Ca^{2+}-ionophore A23187 aggregate platelets in the presence of inhibitors of the first (ADP-mediated) and second (cyclooxygenase-dependent) pathway of platelet activation. This aggregation, via a third pathway, was hypothesized to be mediated by the alkoxyether lipid platelet-activating factor (PAF). We recently demonstrated virtual absence of plasmalogen-type alkoxyether lipids and deficiency in key enzymes of their biosynthesis in Zellweger patients. We hypothesized that PAF synthesis might also be impaired.

We report two Zellweger patients with an undetectable A23187-induced PAF synthesis of leukocytes (patients, < 3 pmol PAF/10^8 granulocytes (PMN); four age-matched controls, 249–2,757 pmol PAF/10^8 PMN; five adult controls, 291–5,433 pmol PAF/10^8 PMN). In a third patient, residual PAF synthesis was detected. However in all patients the thrombin-induced third mechanism of platelet aggregation was present. We therefore conclude that PAF may not be the mediator of the third pathway.

Introduction

Platelet-activating factor (PAF),[1] the alkoxyether lipid 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphocholine (1–3), was originally described as a lipidlike factor originating from IgE-sensitized rabbit basophils that induced rabbit platelet aggregation and release (4). PAF synthesis has since been reported upon appropriate stimulation of a large diversity of cell types, including several animal cell systems (5–11), human cultured lymphoid cell lines (12) and endothelial cells (13, 14), and human basophils (15), neutrophils (15, 16), monocytes (15), and platelets (8, 11, 17). Molecular heterogeneity of PAF exists, however. The rabbit basophil-derived PAF, for instance, is a 9:1 mixture of the 1-O-octadecyl and 1-O-hexadecyl compound (18). It is presently not known whether the different cells synthesize different PAF species.

Platelets can be aggregated by the Ca^{2+}-ionophore A23187, high doses of collagen, or thrombin, in the presence of inhibitors of both the first ADP-mediated pathway and the second cyclooxygenase-dependent pathway of platelet activation (19, 20). A third mechanism of platelet activation was therefore proposed. Because the PAF-induced aggregation of rabbit platelets was shown to be independent of ADP and products of the cyclooxygenase pathway (21), it was hypothesized that PAF might be the mediator of this third pathway (8, 9, 22). However, conflicting results have since been reported regarding the role of ADP and products of the cyclooxygenase pathway in PAF-induced human platelet aggregation. ADP removal resulted in inhibition of irreversible aggregation (23–26) with no effect on the platelet release reaction (23, 24), or it completely inhibited the release (25, 26). Aspirin or indomethacin were found not to inhibit the irreversible aggregation (25) and release (23), and to completely inhibit irreversible aggregation (23–28) and release (24–27). The role of PAF as mediator of the third pathway was also questioned by studies in which platelets that were specifically desensitized to PAF still demonstrated the third pathway (26, 29–32). A direct method to study the role of PAF as the mediator of this third pathway would be to investigate the platelet function of patients with deficient PAF synthesis.

In 1964 Bowen et al. (33) described two patients affected by the cerebro-hepato-renal syndrome of Zellweger. This autosomal recessive disorder is clinically characterized by generalized severe hypotonia, typical craniofacial dysmorphism with a high forehead and large fontanels, hepatomegaly, renal cysts, severe psychomotor retardation, and death usually within the first year of life. Goldfischer et al. (34) first described the absence of peroxisomes from the hepatocytes and renal proximal tubule cells of Zellweger patients. This probably results in the reported disturbances of the metabolism of bile acids (35–37), pipecolic acid (37–40), and very-long-chain fatty acids (41, 42) in this syndrome.

Dihydroxyacetone phosphate acyltransferase (DHAP-AT) and alkyl-DHAP synthase, the key enzymes in the synthesis of alkoxyether lipids, such as plasmalogens and PAF, were shown to be exclusively localized in the microperoxisomes of the rat brain and guinea pig liver (43, 44). Therefore it was proposed that alkoxyether lipid synthesis is defective in Zellweger patients (45). Indeed DHAP-AT and alkyl-DHAP synthase were recently shown to be severely deficient in several tissues and cells of Zellweger patients (46–48). Cultured skin fibroblasts of Zellweger patients were demonstrated to have a severely impaired plasmalogen biosynthesis (49). Plasmalogens were nearly absent in brain, kidney, liver, muscle, and heart tissues and severely re-

Address reprint requests to Augueste Sturk, Academic Medical Center, Department of Hematology, Division of Hemostasis & Thrombosis, F4-209, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.

Received for publication 30 January 1986 and in revised form 9 October 1986.

1. *Abbreviations used in this paper:* DHAP-AT, dihydroxyacetone phosphate acyltransferase; PAF, platelet-activating factor; PMN, polymorphonuclear leukocytes.

J. Clin. Invest.

0021-9738/87/02/0344/07 $1.00
Volume 79, February 1987, 344–350

duced in erythrocytes and fibroblasts of Zellweger patients (50, 51). In view of the reported abnormalities in the alkoxyether lipid metabolism, as confirmed by the plasmalogen findings, we hypothesized that Zellweger patients may also have an impaired PAF synthesis.

In the present study we describe studies with blood cells from three patients with the typical clinical and biochemical features of the Zellweger syndrome. The patients demonstrated severely impaired A23187-induced synthesis of PAF by the leukocyte but normal platelet aggregation. We conclude that PAF may not be the mediator of the third pathway of platelet activation.

Methods

Patients. Three patients were investigated. All patients displayed the typical clinical and biochemical characteristics of the Zellweger syndrome. The diagnosis was confirmed biochemically by the reduced plasmalogen content and DHAP-AT activity of fibroblasts and several tissues.

Patient 1 (S.M.) has been reported before (46, 51). She was a girl from nonconsanguinous, healthy parents of Moroccan origin. PAF synthesis and platelet functions were investigated at 23 d of age. She died at the age of 50 d.

Patient 2 (R.Z.) was a girl from consanguinous Pakistan parents. She was investigated by us at 29 d of age and died 3 d later.

Patient 3 (J.G.) was a girl from nonconsanguinous healthy parents from Cape Verdi. She was investigated by us both at 58 and 62 d of age. She died at the age of 3 mo.

The patients received the regular hospital diet according to age and body weight and did not receive drugs known to interfere with PAF synthesis.

Controls. Four children of similar age to the Zellweger patients and with nonrelated diseases were also investigated. Controls 1 (a Dutch girl), 2 (an Indian boy), 3 (a Dutch boy), and 4 (a Moroccan boy) were studied at 12, 29, 49 and 76 d of age respectively. In each investigation of the platelet function and PAF synthesis of a Zellweger patient or an age-matched control, an adult control was included.

Informed consent was obtained from the adult controls and parents of all children investigated. The study was approved by the medical ethical committee of the Academic Medical Center.

Materials. PAF, lyso-PAF, and A23187 were obtained from Calbiochem-Behring Corp. (La Jolla, CA), soluble bovine tendon collagen from General Diagnostics (Morris Plains, NJ), sodium arachidonate from Bio-Data Corp. (Hatboro, PA), bovine thrombin from Roche Diagnostica (Basel, Switzerland), pig pancreas phospholipase A_2 from Boehringer Mannheim GmbH (Mannheim, FRG), and [^{3}H]-PAF (1-O[alkyl-1′2′-^{3}H]-2-acetyl-sn-glyceryl-3-phosphorylcholine, specific activity 45 Ci/mmol) from New England Nuclear (Boston, MA). Aspégic, the water soluble lysine derivative of acetylsalicylic acid (aspirin), was obtained from Laboratoire Egic (Amilly, France). ADP, human fibrinogen, creatine phosphate, creatine phosphokinase, and essentially fatty acid free bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO).

Isolation of the cells. Due to the limited amounts of blood obtainable from these very young and severely ill patients the procedures for the isolation of the cells had to be optimized and standardized. A single volume of 4.2 ml blood anticoagulated with 0.8 ml acid-citrate-dextrose (52) was centrifuged 8 min at 160 *g*. The platelet-rich plasma was removed and used for platelet isolation by gel filtration in the Ca^{2+}-free Tyrode system of Lages et al. (53). The residual erythrocyte layer was centrifuged for 15 min at 160 *g* to separate lymphocytes and granulocytes (PMN) into the buffy coat and erythrocyte layer. These layers were separately used for the isolation of the leukocytes.

The erythrocytes were lysed by addition of 6 vol of cold 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA. Subsequently the leukocytes were pelleted by 10 min centrifugation at 240 *g*, once washed with cold EDTA–Tyrode (137 mM NaCl, 2.6 mM KCl, 0.36 mM NaH_2PO_4, 5.5 mM glucose, 11.9 mM $NaHCO_3$, 0.25% albumin, 0.5 mM Na_2EDTA, pH 7.35) and washed once with cold Tyrode without EDTA. The leukocytes were finally resuspended in cold Tris–Tyrode (omitting $NaHCO_3$ and EDTA, but including 10 mM Tris, 2 mM $CaCl_2$, and 1 mM $MgCl_2$) and stored on melting ice. Aliquots of the leukocytes of the whole blood and the isolated leukocytes were taken for microscopical subtyping after routine Jenner–Giemsa staining.

Platelet aggregation studies. Platelet aggregation was performed in a dual channel aggregometer (Payton Scientific, Inc., Buffalo, NY) at 37°C with a stirrer speed of 900 rpm in a final volume of 0.4 ml containing $1–2 \times 10^8$ gel-filtered platelets per ml, with (ADP-, collagen-, and PAF-induced aggregation) or without (thrombin-induced aggregation) 1.25 mg fibrinogen per ml. The thrombin-induced aggregations were performed in the absence and presence of 5 mM creatine phosphate and 40 U creatine phosphokinase per ml, as well as 100 μM soluble aspirin to inhibit the first and second pathway of platelet activation respectively. The inhibitor concentrations were sufficient to inhibit aggregation in normal platelet-rich plasma induced by 25 μM ADP and 800 μM sodium arachidonate, which were checked at the start of each investigation of a patient or control.

The concentrations of the gel-filtered platelets of the patients, age-matched controls, and adult controls tested simultaneously were adjusted to comparable concentrations because this variable is known to affect the speed and extent of platelet aggregation.

Synthesis of PAF. PAF synthesis by the isolated leukocytes, suspended in the Tris–Tyrode buffer (137 mM NaCl, 2.6 mM KCl, 0.36 mM NaH_2PO_4, 5.5 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Tris, 0.25% albumin, pH 7.35) was stimulated in the aggregometer by 10 μM A23187 (final volume 0.4 ml, 1×10^7 leukocytes per ml, 900 rpm, 37°C, 45 min). The reaction was stopped by the addition of 1.6 ml ethanol and the sample was stored at −20°C. The sample was thawed, stirred 30 min at room temperature, and centrifuged 20 min at 1500 *g*. The supernatant was removed and evaporated to dryness. The residue was extracted once by addition of 1.0 ml of a 5:10:4 chloroform/methanol/water mixture, mixing, phase separation by addition of 0.3 ml chloroform and 0.3 ml water, and centrifugation. The water layer was extracted twice more with 0.25 ml chloroform. The three chloroform extracts were pooled and evaporated to dryness. The residue was dissolved in 100 μl chloroform and submitted to thin layer chromatography (silica gel 60F 254; E. Merck, Darmstadt, FRG) with a 70:35:7 chloroform/methanol/water mixture as running solvent (8). The components were localized by iodine-vapour staining. PAF (R_f = 0.30) was separated from lyso-PAF (R_f = 0.25), 1-O-alkyl-2-acyl-glyceryl-3-phosphorylcholine (1-alkyl-2-acyl-GPC; R_f = 0.40), A23187 (R_f = 0.80), and arachidonic acid (R_f = 0.95). 1-cm zones of the plates were scraped and the silica gel was extracted six times with 80% ethanol. The combined ethanol extracts were dried and the residue was redissolved in 100 μl ethanol and used for the PAF quantification.

The PAF quantification was performed by comparison of PAF-induced aggregation measurements of rabbit platelets, isolated by gel filtration as described above (53). Aggregations were performed in the presence of 1.25 mg fibrinogen per ml and, unless indicated otherwise, in the absence of the inhibitors of the first and second pathway. Rabbit platelets were used because they were found to be at least 10-fold more sensitive to PAF than human platelets, as has been reported before (22). The gel-filtered rabbit platelet system had a detection limit of 0.01–0.08 pmol PAF, whereas in the adult controls 25–400 pmol PAF were measured. The system was therefore considered sufficiently sensitive to detect even low levels of PAF synthesis. The isolated leukocytes consisted of PMN and lymphocytes. In contrast to PMN, lymphocytes do not synthesize PAF (15). Therefore, PAF synthesis was expressed as pmol per 10^8 PMN.

PAF was characterized by R_f on thin layer chromatography, inactivation by incubation with phospholipase A_2 from pig pancreas (54) and residual platelet aggregation in the presence of creatine phosphate/creatine phosphokinase and aspirin. The identity of PAF was confirmed by conversion to lyso-PAF (0.03 N NaOH, 15 min 37°C, yield > 95%), chloroform extraction, and reacetylation to PAF (1 part chloroform plus 5 parts acetic anhydride, 2 h, 37°C, yield > 67%).

After this work was completed we reinvestigated the time course of the PAF synthesis. The synthesis proved maximal after 15 min of A23187-induced stimulation, and after 45 min 40% of the biological PAF activity was still present.

Results

Isolation of the cells. The overall recovery of the gel-filtered platelets from the blood samples was similar in patients, age-matched controls and the adult controls tested simultaneously (range 30–50%). The results of the isolation of the leukocytes are presented in Table I. The total recoveries of the leukocytes from buffycoat and erythrocyte layer compared with the blood cell count were 26–67% in the Zellweger patients, 70–86% in the age-matched controls, and 36–90% in adult controls, respectively. The isolated leukocytes were mainly PMN and lymphocytes. Compared with the whole blood count, the isolated leukocytes from the erythrocyte layers were enriched in PMN and those from the buffycoats were depleted of PMN.

Synthesis of PAF. PAF synthesis by leukocytes from one adult control varied considerably on 13 different occasions (mean 1,629, range 325–5,433 pmol/10^8 PMN); also between five adult controls a substantial variation was noticed (25 determinations, mean 1,759, range 291–5,433 pmol PAF/10^8 PMN). The PAF synthesis of the four age-matched controls was within the adult control range. However, PAF synthesis of patients 1 and 2 was undetectable and at least 100-fold lower than the range of the adult controls (Table II). Patient 3, investigated at the age of 58 d, also demonstrated undetectable PAF synthesis (< 3 pmol/10^8 PMN) in the rabbit platelet aggregation system routinely used, i.e., without inhibitors of first and second pathway. When investigated at the age of 62 d, however, PAF synthesis was detectable in the routine rabbit platelet system (110 pmol PAF/10^8 PMN) but undetectable when the inhibitors were added (< 3 pmol PAF/10^8 PMN) (Table II). The adult control demonstrated similar PAF synthesis when tested in the presence and absence of the inhibitors (610 and 541 pmol PAF/10^8 PMN, respectively).

These results indicate a defect in PAF biosynthesis in the patients, but two alternative explanations cannot be excluded. First, the PAF synthesis by the leukocytes of the Zellweger patients might have been undetectable due to an increased cellular breakdown of PAF to 1-alkyl-2-acyl-GPC, which might have played a role especially at a low residual level of PAF synthesis. To investigate this possibility 10 nCi [^{3}H]-PAF was added to leukocytes isolated from three adult controls and patient 2, just before the A23187 stimulation. This amount of PAF (0.22 pmol) was equivalent to 5.5 pmol PAF/10^8 leukocytes, i.e., 50-fold lower than the control range of PAF synthesis. In a total of eight experiments with the leukocytes of the adult controls 43–72% (mean 60%) of the radioactivity added as [^{3}H]-PAF was recovered in the PAF isolation procedure. Of this recovered radioactivity 19–49% (mean 30%) was located in the zone of the thin layer chromatogram corresponding to PAF, next to the radioactivity in the 1-alkyl-2-acyl-GPC zone. With the leukocytes of patient 2, 73% of the radioactivity was recovered and 22% of this radioactivity was located in the PAF zone. This indicated that the PAF recovery was normal and increased cellular breakdown of PAF by the leukocytes of the patients unlikely, even at the low PAF level used.

Second, an inhibitor of the PAF synthesis might have been present in the leukocytes of the patient. To investigate the possibility that this inhibitor was excreted, mixing experiments were performed in which 1:1 mixtures of the leukocytes isolated from the erythrocyte layers of adult controls with those of patients 2 and 3 (at 58 d of age) were stimulated with A23187. Intermediate PAF synthesis was obtained (adult control 1030, patient 2, < 3, mixture 440 pmol PAF/10^8 PMN; adult control 905, patient 3, < 3, mixture 290 pmol PAF/10^8 PMN). This indicates that the presence of an extracellular inhibitor was also unlikely, but of course does not exclude the possibility of an intracellular inhibitor not secreted by the leukocytes of the patients.

Platelet aggregation studies. Results of the aggregation studies

Table I. Isolation of the Leukocytes

	% Recovery		% PMN		
	Ery	BC	WB	Ery	BC
Patient 1	41 (59)	26 (18)	38 (76)	64 (90)	25 (10)
Patient 2	23 (26)	10 (10)	63 (62)	75 (80)	19 (38)
Patient 3*	14 (31)	12 (17)	24 (57)	37 (88)	9 (24)
Patient 3‡	46 (49)	11 (19)	33 (66)	37 (82)	1 (26)
Control 1	32 (42)	39 (25)	44 (62)	47 (79)	4 (11)
Control 2	50 (71)	36 (19)	23 (68)	41 (87)	16 (26)
Control 3	45 (48)	25 (18)	44 (57)	52 (87)	8 (32)
Control 4	26 (59)	48 (17)	32 (52)	50 (74)	2 (23)

Leukocytes of patients, age-matched controls, and adult controls were isolated from the buffycoat (*BC*) and erythrocyte layer (*Ery*) as described in Methods. The percentage recovery of the leukocytes isolated from the buffycoat and the erythrocyte layer are each presented relative to the initial whole blood count. Percent PMN represents the percentage granulocytes in the whole blood (*WB*) or in leukocytes isolated from the buffycoat or the erythrocyte layer. The results obtained with the adult controls tested simultaneously with a patient or age-matched control are given in parentheses. Patient 3 was investigated at 58 (*) and 62 (‡) d of age.

Table II. PAF Synthesis of the Leukocytes

	Ery	BC
Patient 1	<2 (1050)	<2 (1000)
Patient 2	<3 (1030)	<13 (490)
Patient 3*	<3 (905)	<56 (868)
Patient 3‡−	110 (541)	<1000 (1057)
Patient 3‡+	<3 (610)	— —
Control 1	2305 (2380)	249 (484)
Control 2	1522 (3718)	2757 (5052)
Control 3	1025 (875)	1333 (469)
Control 4	992 (1586)	1742 (2568)

Leukocytes were isolated from the erythrocyte layer (*Ery*) and buffycoat (*BC*) of patients, age-matched controls, and adult controls tested simultaneously, and PAF synthesis was induced by 10 μM A23187 according to Methods. Patient 3 was investigated at 58 (*) d and 62 (‡) d of age. PAF synthesis at 62 d of age was quantified in the absence (−) and presence (+) of inhibitors of first and second pathway. Results are presented as pmol PAF/10^8 PMN. If the PAF synthesis was undetectable, the lower detection limits of the assay as determined by the PAF sensitivity of the batch of rabbit platelets and the percent granulocytes of that particular leukocyte preparation are given. The values of the adult controls tested simultaneously are given in parentheses.

of the gel-filtered platelets of the three patients and the adult controls tested simultaneously are presented in Fig. 1. Due to the low concentration of the gel-filtered platelets of the patients and adult controls tested simultaneously, only up to 65% maximal aggregation was obtained. In the absence of the inhibitors platelets from the patients and adult controls aggregated similarly upon stimulation by ADP, collagen, or thrombin, although patients 1 and 3 demonstrated a somewhat reduced response to thrombin. In the presence of the inhibitors of the first and second pathways, thrombin induced a platelet aggregation in both adult controls and patients. This aggregation again appeared somewhat reduced in patients 1 and 3 but was still clearly present.

Platelets from patient 2, as well as adult controls, aggregated irreversibly with 10 nM PAF. Patient 3 was tested only at 100 nM PAF, which induced irreversible aggregation. Patient 1 was not tested with PAF.

Discussion

In the present study the leukocytes of the normal adult controls and the four age-matched controls demonstrated easily detectable PAF synthesis (291–5,433 pmol/10^8 PMN). These data are in close agreement with the PAF synthesis reported by Oda et al. (55) (range 140–2,230 pmol PAF/10^8 PMN). Two Zellweger patients are described with an undetectable, at least 100-fold reduced, PAF synthesis (< 3 pmol PAF/10^8 PMN) of the isolated leukocytes. In a third Zellweger patient, evidence of some residual PAF synthesis was obtained. However, the gel-filtered platelets isolated from the same blood samples demonstrated normal platelet aggregation and a normal thrombin-induced third pathway. In our opinion this strongly suggests that PAF is not the mediator of the third pathway.

It may be argued that the assay for PAF synthesis was suboptimal and might partly explain the undetectable PAF synthesis. Indeed PAF synthesis under our conditions proved maximal after 15 min of leukocyte stimulation. But although the difference in PAF synthesis between 15 and 45 min stimulation is substantial, i.e., after 45 min 40% of the maximal PAF synthesis is present, it is insignificant in relation to the 100-fold difference in PAF synthesis observed between the two Zellweger patients and control subjects. Also, in each investigation of a patient an adult control was tested simultaneously. This possibility is therefore unlikely.

It may also be argued that a defective PAF synthesis by the leukocyte is not indicative of a defective synthesis in the platelet. But a general defect in the alkoxyether lipid metabolism has been demonstrated in all tissues and cells of Zellweger patients tested, including liver, brain, kidney, muscle, heart, leukocytes,

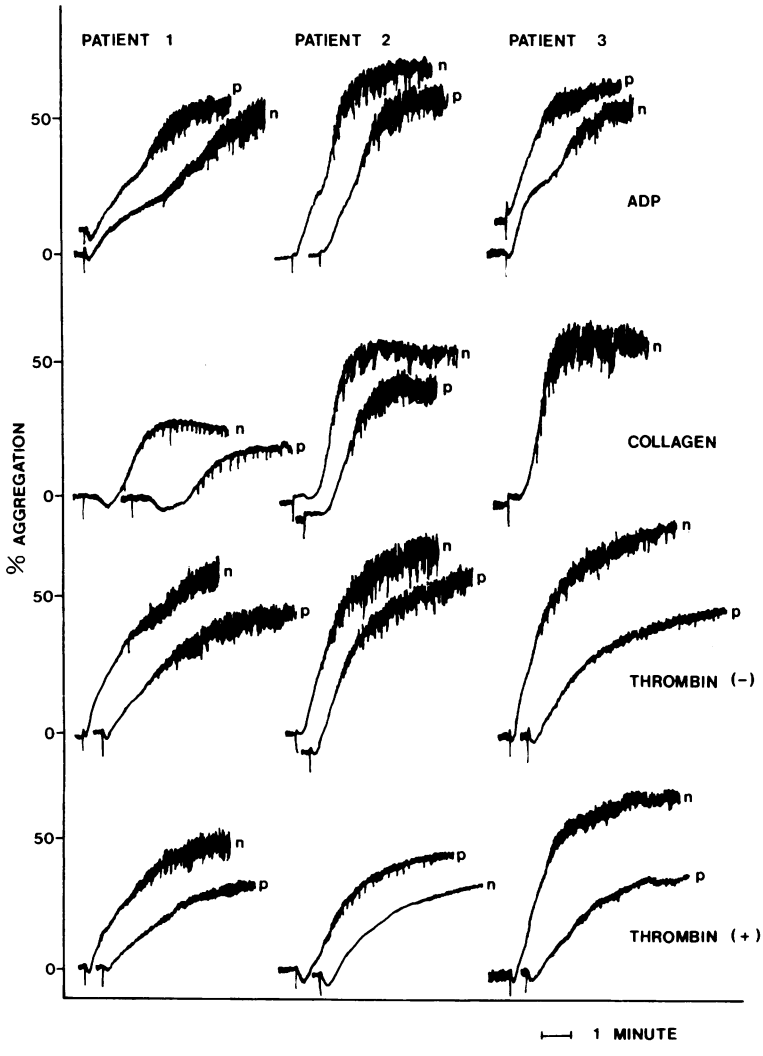

Figure 1. Platelet aggregation studies. The aggregation studies of the gel-filtered platelets of patients 1–3 (*p*), and the adult controls tested simultaneously (*n*) were performed as described in Methods. The concentrations used were 1 μM ADP (patients 1–3), 0.8 μg collagen per ml (patients 1, 2; an insufficient amount of platelets was obtained from patient 3), 0.1 U thrombin per ml (patients 1, 3) or 0.05 U thrombin per ml (patient 2) in the absence (–) or presence (+) of creatine phosphate/creatine phosphokinase and aspirin. The final concentrations of the gel-filtered platelets during the aggregations were 1.8, 1.8, and 1.65 × 10^8 platelets per ml in patients/controls 1, 2, and 3 respectively. The aggregations of the platelets of the patients were similar to the controls and the third mechanism of platelet activation, i.e., thrombin-induced aggregation in the presence of the inhibitors, was clearly present.

and fibroblasts (46–51). The deficient PAF synthesis observed with the leukocytes of the patients is therefore also likely to exist in the platelet. Moreover, PAF synthesis by normal platelets is extremely low. Marcus et al. (25) demonstrated 23 pmol PAF synthesized by 2.5 μM A23187 stimulation of platelets isolated from 8 U of human blood, which would be equivalent to < 0.1 pmol PAF/5 $\times 10^8$ platelets. Alam et al. (11), using 50 $\times 10^8$ platelets and stimulated with the same A23187 concentration, found 0.3–3.9 pmol PAF/5 $\times 10^8$ platelets. PAF synthesis by such platelet suspensions, however, may mostly be due to leukocyte contamination (56). By blocking the acetyl hydrolase involved in the breakdown of PAF to lyso-PAF, Touqui et al. (57) reported thrombin-induced synthesis of 6 pmol PAF by 5 $\times 10^8$ platelets, but the effect of thrombin or the blocking of the acetyl hydrolase on contaminating leukocytes was not mentioned. In view of the low reported PAF synthesis by platelets, which does not enable distinction between a reduced and a deficient synthesis, the possible interference of leukocytes and the need to use all isolated platelets (4.6–6.0 $\times 10^8$ cells in the 3 patients) for either a single platelet PAF synthesis determination or the platelet aggregation studies presented, we decided that platelet PAF synthesis studies were not feasible in our patients.

The Zellweger patients were from Morocco, Pakistan, and Cape Verdi. To exclude the possibility that deficient PAF synthesis might be due to regional variations instead of the Zellweger syndrome, we investigated age-matched controls 2 and 4 from India and Morocco respectively. In these controls a normal PAF synthesis was found.

In studies with human PMN, 30–40% of the total PAF synthesized was reported to be released (58). Also, 60% of the PAF added to a suspension of rabbit PMN was reported to be converted within 5 min into inactive 1-alkyl-2-acyl-GPC (59). In our experiments we measured PAF synthesis after 45 min stimulation of the PMN. It might therefore be argued, that the undetectable PAF synthesis of the patients might in fact be due to an increased conversion of PAF into inactive 2-acyl compounds. However, the [^{3}H]-PAF recovery and distribution of radioactivity across PAF and 1-alkyl-2-acyl-GPC zones of the chromatogram of patient 2 were normal. Also the mixing experiments in patients 2 and 3 did not indicate an increased degradation of PAF. Taking the reported abnormalities of the synthesis of the 1-O-alkoxyether lipids in the Zellweger syndrome into account (46–51), the explanation of the undetectable synthesis by increased conversion into a 2-acyl compound seems even more unlikely.

Still, some caution is necessary. In all Zellweger cells and tissues investigated, some residual DHAP-AT and alkyl-DHAP synthase activities were detectable (46–48), the plasmalogens were nearly absent in most tissues but less reduced in leukocytes, erythrocytes, and cultured fibroblasts (50, 51), and also the cultured fibroblasts demonstrated residual de novo plasmalogen biosynthesis (49). They may have microperoxisomes albeit in a severely reduced amount (60). It can therefore not be completely excluded that some residual PAF synthesis by the platelet, sufficient to warrant the third pathway, is present in patients 1 and 2. In support of this possibility, the Zellweger patient 3 initially demonstrated an undetectable PAF synthesis. This defect seemed not to be present in the blood samples collected a few days later. On this latter occasion the PAF synthesis of this patient was found to be detectable in the routinely used rabbit platelet aggregation system, i.e., without inhibitors of first and second pathway, but undetectable in the presence of the inhibitors. The discrepancy may be explained by synergistic effects in the rabbit platelet aggregation system of low, subthreshold levels of ADP and PAF (61). Small quantities of ADP are likely to be released or leak from the rabbit platelet suspension upon storage during the PAF quantification. A low amount of PAF, insufficient to cause platelet aggregation by itself, might then suffice to be detected in the rabbit platelet aggregation quantification by synergism with ADP. Addition of inhibitors, however, would inhibit this synergistic detection. In patients 1 and 2 even this amount of PAF synthesis could then not be demonstrated.

Evidently, we cannot exclude some residual PAF synthesis, sufficient to mediate the third pathway. Also, the results show that two of the three patients had reduced platelet responses to thrombin (plus inhibitors), although they had normal responses to ADP. The results, then, might be interpreted as favoring PAF involvement in certain aggregation responses. Particularly if stimulus-response coupling is viewed not in an all-or-none fashion, but rather as mediated by several pathways any one of which can be ablated to produce a still partially responsive cell. Studies comparing dose-response effects in patients and normals as well as the effect of stimuli other than thrombin are needed before definite conclusions can be made. On balance, however, our data support earlier observations (26, 29–32) that PAF may not be the mediator of the third pathway.

Acknowledgments

The authors are grateful to Prof. H. van den Bosch (Utrecht), Prof. L. A. H. Monnens, Dr. J. M. F. Trijbels, and Dr. L. C. P. Govaerts (Nijmegen), Dr. P. Nelck (Assen), and Dr. R. J. A. Wanders (Amsterdam) for their helpful discussions during these experiments. We gratefully acknowledge Dr. G. P. A. Smit (Groningen) for his permission to study patient R.Z.

References

1. Demopoulos, C. A., R. N. Pinckard, and D. J. Hanahan. 1979. Platelet-activating factor. Evidence for 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine as the active component (a new class of lipid chemical mediators). *J. Biol. Chem.* 254:9355–9358.

2. Benveniste, J., M. Tence, P. Varenne, J. Bidault, C. Boullet, and J. Polonsky. 1979. Semi-synthèse et structure proposée du facteur activant les plaquettes (PAF): PAF-Acether, un alkyl ether analogue de la lysophosphatidylcholine. *C.R. Acad. Sci. Ser. III Sci. Vie.* 289:1037–1040.

3. Blank, M. L., F. Snyder, L. W. Byers, B. Brooks, and E. E. Muirhead. 1979. Antihypertensive activity of an alkyl ether analog of phosphatidylcholine. *Biochem. Biophys. Res. Commun.* 90:1194–1200.

4. Benveniste, J., P. M. Henson, and C. G. Cochrane. 1972. Leukocyte-dependent histamine release from rabbit platelets: the role of IgE, basophils, and a platelet-activating factor. *J. Exp. Med.* 136:1356–1377.

5. Mencia-Huerta, J. M., and J. Benveniste. 1979. Platelet-activating factor and macrophages. I. Evidence for the release from rat and mouse peritoneal macrophages and not from mastocytes. *Eur. J. Immunol.* 9: 409–415.

6. Pirotzky, E., E. Ninio, J. Bidault, A. Pfister, and J. Benveniste. 1984. Biosynthesis of platelet-activating factor and acetyltransferase activity in isolated rat kidney cells. *Lab. Invest.* 51:567–572.

7. Lynch, J. M., G. Z. Lotner, S. J. Betz, and P. M. Henson. 1979. The release of platelet-activating factor by stimulated rabbit neutrophils. *J. Immunol.* 123:1219–1226.

8. Chignard, M., J. P. Le Couedic, M. Tencé, B. B. Vargaftig, and J. Benveniste. 1979. The role of platelet-activating factor in platelet aggregation. *Nature (Lond.).* 179:799–800.

9. Chignard, M., J. P. Le Couedic, B. B. Vargaftig, and J. Benveniste. 1980. Platelet-activating factor (Paf-acether) secretion from platelets: effects of aggregating agents. *Brit. J. Haematol.* 46:455–464.

10. Namm, D. H., and J. A. High. 1980. Thrombin-induced formation of a polar lipid material within platelets that possesses platelet-activating activity. *Thromb. Res.* 20:285–292.

11. Alam, I., J. B. Smith, and M. J. Silver. 1983. Human and rabbit platelets form platelet activating factor in response to calcium ionophore. *Thromb. Res.* 30:71–79.

12. Bussolino, F., R. Foa, F. Malavasi, M. L. Ferrando, and G. Camussi. 1984. Release of platelet-activating factor (PAF)-like material from human lymphoid cell lines. *Exp. Hematol. (NY).* 12:688–693.

13. Camussi, G., M. Aglietta, F. Malavasi, C. Tetta, W. Piacibello, F. Sanavio, and F. Bussolino. 1983. Release of platelet-activating factor from human endothelial cells in culture. *J. Immunol.* 131:2397–2403.

14. Prescott, S. M., G. A. Zimmerman, and T. M. McIntyre. 1984. Human endothelial cells in culture produce platelet-activating factor (1-alkyl-2-acetyl-sn-glycero-3-phosphocholine) when stimulated with thrombin. *Proc. Natl. Acad. Sci. USA.* 81:3534–3538.

15. Camussi, G., M. Aglietta, R. Coda, F. Bussolino, W. Piacibello, and C. Tetta. 1981. Release of platelet-activating factor (PAF) and histamine. II. The cellular origin of human PAF: monocytes, polymorphonuclear neutrophils and basophils. *Immunology.* 42:191–199.

16. Lotner, G. Z., J. M. Lynch, J. S. Betz, and P. M. Henson. 1980. Human neutrophil derived platelet-activating factor. *J. Immunol.* 124: 676–684.

17. Chap, H., G. Mauco, M. F. Simon, J. Benveniste, and L. Douste-Blazy. 1981. Biosynthetic labelling of platelet-activating factor from radioactive acetate by stimulated platelets. *Nature (Lond.).* 289:312–314.

18. Hanahan, D. J., C. A. Demopoulos, J. Liehr, and R. N. Pinckard. 1980. Identification of platelet activating factor isolated from rabbit basophils as acetyl glyceryl ether phosphorylcholine. *J. Biol. Chem.* 255: 5514–5516.

19. Kinlough-Ratbone, R. L., M. A. Packham, H. J. Reimers, J. P. Cazenave, and J. F. Mustard. 1977. Mechanisms of platelet shape change, aggregation and release induced by collagen, thrombin or A23187. *J. Lab. Clin. Med.* 90:707–719.

20. Packham, M. A., M. A. Guccione, J. P. Greenberg, R. L. Kinlough-Ratbone, and J. F. Mustard. 1977. Release of ^{14}C-serotonin during initial platelet changes induced by thrombin, collagen or A23187. *Blood.* 50:915–926.

21. Cazenave, J. P., J. Benveniste, and J. F. Mustard. 1979. Aggregation of rabbit platelets by platelet-activating factor is independent of the release reaction and the arachidonate pathway and inhibited by membrane-active drugs. *Lab. Invest.* 41:275–285.

22. Vargaftig, B. B., M. Chignard, and J. Benveniste. 1981. Present concepts on the mechanisms of platelet aggregation. *Biochem. Pharmacol.* 30:263–271.

23. McManus, L. M., D. J. Hanahan, and R. N. Pinckard. 1981. Human platelet stimulation by acetyl glyceryl ether phosphorylcholine. *J. Clin. Invest.* 67:903–906.

24. Chesney, C. M., D. D. Pifer, L. W. Byers, and E. E. Muirhead. 1982. Effect of platelet-activating factor (PAF) on human platelets. *Blood.* 59:582–585.

25. Marcus, A. J., L. B. Safier, H. L. Ullman, K. T. H. Wong, M. J. Broekman, B. B. Weksler, and K. L. Kaplan. 1981. Effects of acetyl-glyceryl-ether phosphorylcholine on human platelet function in vitro. *Blood.* 58:1027–1031.

26. Kloprogge, E., G. H. de Haas, G. Gorter, and J. W. N. Akkerman. 1983. Stimulus-response coupling in human platelets. Evidence against a role of PAF-Acether in the "third pathway". *Thromb. Res.* 30:107–112.

27. MacIntyre, D. E., A. M. Shaw, W. K. Pollock, G. Marks, and J. Westwick. 1983. Role of endogenous arachidonate metabolites in phospholipid-induced human platelet activation. *Adv. Prostaglandin Thromboxane Leukotriene Res.* 11:423–428.

28. Rao, G. H. R., H. H. O. Schmid, K. R. Reddy, and J. G. White. 1982. Human platelet activation by an alkylacetyl analogue of phosphatidylcholine. *Biochim. Biophys. Acta.* 715:205–214.

29. Hornby, E. J., and C. R. Perry. 1983. 1-O-hexadecyl-2-acetyl-sn-3-glycerophosphorylcholine (PAF): some effects on the aggregation of human platelets by thrombin or collagen. *Thromb. Haemostasis* 50: 586–587.

30. Nunn, B. 1983. Total and selective desensitization of human platelets to synthetic platelet activating factor (PAF): evidence that extracellular PAF does not mediate collagen-induced aggregation. *Thromb. Res.* 31:657–663.

31. Hallam, T. J., M. C. Scrutton, and R. B. Wallis. 1983. The role of 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (AcGEPC) and palmitoyllysophosphatidate in the responses of human blood platelets to collagen and thrombin. *FEBS (Fed. Eur. Biochem. Soc.) Letters* 162: 142–146.

32. Wal, F., D. Joseph, L. Touqui, and B. B. Vargaftig. 1985. PAF-Acether may not mediate the third pathway of platelet aggregation since self-desensitization reduces the effects of low thrombin but enhances those of convulxin. *Thromb. Haemostasis.* 53:99–104.

33. Bowen, P., C. S. N. Lee, H. Zellweger, and R. Lindenberg. 1964. A familial syndrome of multiple congenital defects. *Bull. Johns Hopkins Hosp.* 114:402–414.

34. Goldfischer, S., C. L. Moore, A. B. Johnson, A. J. Spiro, M. P. Valsamis, H. K. Wisniewski, R. H. Ritch, W. T. Norton, I. Rapin, and L. M. Gartner. 1973. Peroxisomal and mitochondrial defects in the cerebro-hepato-renal syndrome. *Science (Wash. DC).* 182:62–64.

35. Hanson, R. F., P. Szczepanik-van Leeuwen, G. C. Williams, G. Grabowski, and H. L. Sharp. 1979. Defects of bile acid synthesis in Zellweger's syndrome. *Science (Wash. DC).* 203:1107–1108.

36. Monnens, L., J. Bakkeren, G. Parmentier, G. Janssen, U. van Haelst, R. Trijbels, and H. Eyssen. 1980. Disturbances in the bile acid metabolism of infants with the Zellweger (cerebro-hepato-renal) syndrome. *Eur. J. Pediatr.* 133:31–35.

37. Mathis, R. K., J. B. Watkins, P. Szczepanik-van Leeuwen, and I. T. Lott. 1980. Liver in the cerebro-hepato-renal syndrome: detective bile acid synthesis and abnormal mitochondria. *Gastroenterology.* 79: 1311–1317.

38. Danks, D. M., P. Tippett, C. Adams, and P. Campbell. 1975. Cerebro-hepato-renal syndrome of Zellweger. A report of eight cases with comment upon the incidence, the liver lesion, and a fault in pipecolic acid metabolism. *J. Pediatr.* 86:382–387.

39. Trijbels, J. M. F., L. A. H. Monnens, J. A. J. M. Bakkeren, A. H. J. van Raay-Selten, and J. M. B. Corstiaensen. 1979. Biochemical studies in the cerebro-hepato-renal syndrome of Zellweger: a disturbance in the metabolism of pipecolic acid. *J. Inherited Metab. Dis.* 2:39–42.

40. Govaerts, L., L. Monnens, W. Tegelaers, F. Trijbels, and A. van Raay-Selten. 1982. Cerebro-hepato-renal syndrome of Zellweger: clinical symptoms and relevant laboratory findings in 16 patients. *Eur. J. Pediatr.* 139:125–128.

41. Brown, F. R., A. J. McAdams, J. W. Cumming, R. Konkol, I. Singh, A. B. Moser, and H. W. Moser. 1982. Cerebro-hepato-renal (Zellweger) syndrome and neonatal adrenoleukodystrophy: similarities in phenotype and accumulation of very long chain fatty acids. *Johns Hopkins Med. J.* 151:344–361.

42. Moser, A. E., I. Singh, F. R. Brown, G. I. Solish, R. I. Kelly, P. J. Benke, and H. W. Moser. 1984. The cerebrohepatorenal (Zellweger) syndrome. Increased levels and impaired degradation of very-long-chain fatty acids and their use in prenatal diagnosis. *N. Engl. J. Med.* 310: 1141–1146.

43. Hajra, A. K., C. L. Burke, and C. L. Jones. 1979. Subcellular localization of acylcoenzyme A: dihydroxyacetone phosphate acyltransferase in rat liver peroxisomes (microbodies). *J. Biol. Chem.* 254:10896–10900.

44. Hajra, A. K., and J. E. Bishop. 1982. Glycerolipid biosynthesis in peroxisomes via the acyl dihydroxyacetone phosphate pathway. *Ann. N.Y. Acad. Sci.* 386:170–182.

45. Borst, P. 1983. Animal peroxisomes (microbodies), lipid biosynthesis and the Zellweger syndrome. *Trends Biochem. Sci.* 8:269–272.

46. Schutgens, R. B. H., G. J. Romeyn, R. J. A. Wanders, H. van den Bosch, G. Schrakamp, and H. S. A. Heymans. 1984. Deficiency of acyl-CoA: dihydroxyacetone phosphate acyltransferase in fibroblasts from

patients with Zellweger (cerebro-hepato-renal) syndrome. *Biochem. Biophys. Res. Commun.* 120:179–184.

47. Datta, N. S., G. N. Wilson, and A. K. Hajra. 1984. Deficiency of enzymes catalyzing the biosynthesis of glycerol-ether lipids in Zellweger syndrome. *N. Engl. J. Med.* 311:1080–1083.

48. Schrakamp, G., C. F. P. Roozenboom, R. B. H. Schutgens, R. J. A. Wanders, H. S. A. Heymans, J. M. Tager, and H. van den Bosch. 1985. Alkyl dihydroxyacetone phosphate synthase in human fibroblasts and its deficiency in Zellweger syndrome. *J. Lipid Res.* 26:867–873.

49. Schrakamp, G., R. B. H. Schutgens, R. J. A. Wanders, H. S. A. Heymans, J. M. Tager, and H. van den Bosch. 1985. The cerebro-hepato-renal (Zellweger) syndrome. Impaired de novo biosynthesis of plasmalogens in cultured skin fibroblasts. *Biochim. Biophys. Acta.* 833:170–174.

50. Heymans, H. S. A., R. B. H. Schutgens, R. Tan, H. van den Bosch, and P. Borst. 1983. Severe plasmalogen deficiency in tissues of infants without peroxisomes (Zellweger syndrome). *Nature (Lond.).* 306: 69–70.

51. Heymans, H. S. A., H. van den Bosch, R. B. H. Schutgens, W. H. H. Tegelaers, J. U. Walther, J. Müller-Höcker, and P. Borst. 1984. Deficiency of plasmalogens in the cerebro-hepato-renal (Zellweger) syndrome. *Eur. J. Pediatr.* 142:10–15.

52. Aster, R. H., and J. H. Jandl. 1964. Platelet sequestration in man. I. Methods. *J. Clin. Invest.* 43:843–855.

53. Lages, B., M. C. Scrutton, and H. Holmsen. 1975. Studies on gel-filtered human platelets: isolation and characterization in a medium containing no added Ca^{2+}, Mg^{2+} or K^{+}. *J. Lab. Clin. Med.* 85:811–825.

54. Benveniste, J., J. P. Le Couedic, J. Polansky, and M. Tence. 1977. Structural analysis of purified platelet-activating factor by lipases. *Nature (Lond.).* 269:170–171.

55. Oda, M., K. Satouchi, K. Yasunaya, and K. Saito. 1985. Molecular species of platelet-activating factor generated by human neutrophils challenged with ionophore A23187. *J. Immunol.* 134:1090–1093.

56. Oda, M., K. Satouchi, K. Yasunaga, and K. Saito. 1985. Production of platelet-activating factor by washed rabbit platelets. *J. Lipid Res.* 26:1294–1299.

57. Touqui, L., M. Hatmi, and B. B. Vargaftig. 1985. Human platelets stimulated by thrombin produce platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) when the degrading enzyme acetyl hydrolase is blocked. *Biochem J.* 229:811–816.

58. Ludwig, J. C., L. M. McManus, P. O. Clark, D. J. Hanahan, and R. N. Pinckard. 1984. Modulation of platelet-activating factor (PAF) synthesis and release from human polymorphonuclear leukocytes (PMN): role of extracellular Ca^{2+}. *Arch. Biochem. Biophys.* 232:102–110.

59. Chilton, F. H., J. T. O'Flaherty, J. M. Ellis, C. L. Swendsen, and R. L. Wykle. 1983. Metabolic fate of platelet-activating factor in neutrophils. *J. Biol. Chem.* 258:6357–6361.

60. Arias, J. A., A. B. Moser, and S. L. Goldfischer. 1985. Ultrastructural and cytochemical demonstration of peroxisomes in cultured fibroblasts from patients with peroxisomal deficiency disorders. *J. Cell Biol.* 100:1789–1792.

61. Sturk, A., G. M. Asyee, M. C. L. Schaap, M. van Maanen, and J. W. ten Cate. 1986. Synergistic effects of platelet-activating factor and other platelet agonists in human platelet aggregation and release: the role of ADP and products of the cyclooxygenase pathway. *Thromb. Res.* 40: 359–372.