Platelet Secretion Defect in Patients With the Attention Deficit Disorder and Easy Bruising

By Kazuo Koike, A. Koneti Rao, Holm Holmsen, and Peter S. Mueller, with technical assistance of Janet Willis and Cheryl Beckett

Platelet function was evaluated in 12 patients with the attention deficit disorder and lifelong history of easy bruising. Aggregation and $^{3}H$-serotonin secretion studies in platelet-rich plasma in response to adenosine diphosphate (ADP), epinephrine, and arachidonic acid did not reveal striking abnormalities. Secretion of adenosine triphosphate (ATP), ADP, $\beta$-hexosaminidase, and $\beta$-glucuronidase by gel-filtered platelets in response to the divalent cation ionophore A23187 and low concentrations of thrombin ($\leq 0.1$ U/ml) was impaired in patients as compared to normals. The aggregation response to A23187 (4 $\mu$M) was absent in 8 of the 12 patients. The total stores of the secretable constituents, the retention of incorporated $^{3}H$-serotonin, and the arachidonate metabolism of the platelets were normal. Our findings suggest a new platelet disorder with impaired secretion mechanism, without storage pool deficiency or impaired arachidonate metabolism. The secretion defect in platelets represents a tissue disorder in a functional psychiatric disease. We refocus attention on the role of platelets as a model for neurons in functional disorders, with emphasis on secretion mechanisms rather than amine uptake, storage, and metabolism.

ATTENTION DEFICIT DISORDER (ADD), previously referred to as hyperkinesis, minimal brain dysfunction, or hyperactivity, is a set of behavioral disorders characterized by impaired concentration, easy distractability, learning disorders, hyperkinesis, various neurologic signs, mood instability, and impulsivity. Recent genetic studies have emphasized the hereditary nature of this disorder. Although ADD was originally relegated to childhood and adolescence, recent reports show that adults may manifest residual features of ADD that are characterized by depression and sociopathy, but not hyperactivity. One of us (P.S.M.), in the practice of neuropsychiatry, has noted that (1) the majority of the female adult patients with ADD had a lifelong history of easy bruising, (2) male patients had easy bruising in childhood but not after adolescence, and (3) the history of easy bruising and ADD were tightly linked in families. To determine the mechanisms underlying the bruising tendency, detailed platelet function studies, with particular reference to secretion mechanisms, granule stores, aggregation, and arachidonate metabolism, were carried out on 12 patients. These studies illustrate a novel platelet disorder characterized by a secretion defect without storage pool deficiency or impaired arachidonate metabolism and a hitherto undefined tissue defect in the functional disorder, ADD. Platelets have been previously considered as a model for neurons in functional psychiatric illness. We refocus attention on this concept, with emphasis on the secretory (exocytotic) mechanisms in platelets, rather than on the monoamine metabolism, uptake, and storage.

MATERIALS AND METHODS

Subjects

Twelve patients with ADD, ranging in age from 11 to 55 yr (mean 36 yr), were studied. Except for an 11-yr-old boy with a severe learning disorder who was the son of one of the other patients studied, all other ADD patients were unrelated adult females. They all had problems of distractability and lack of concentration extending back to childhood and a history in childhood of learning problems, and usually, hyperactivity. They all had normal intelligence and did not show signs of schizophrenia or major affective disorders. The diagnosis was substantiated by extensive neuropsychologic testing, including the Halstead-Reitan Battery. All patients had a history of easy bruising, which appeared to be variable in severity. At least five patients volunteered a history of developing spontaneous bruises without definite knowledge of trauma, and in several patients, ecchymotic lesions were noted during the physical examination. A history of menorrhagia was obtained in five patients, with one patient having had a hysterectomy. In two patients there had been excessive bleeding following tonsillectomy in childhood, which required additional medical intervention. Likewise a history of excessive bleeding following dental extractions was elicited in two patients. However, none of the patients had a history of previous blood transfusions. The patients were physically well and had not taken any medications, particularly nonsteroidal antiinflammatory drugs, for 10 days prior to blood withdrawal. Normal subjects were concurrently studied. They were laboratory personnel (mean age 32 yr, range 25–42 yr) without known bleeding or psychiatric disorders and who had not taken any medication for 10 days before blood withdrawal. They were equally distributed between the two sexes.

An informed consent was obtained from both normal subjects and ADD patients.

Chemicals

Thrombin (topical, bovine, Parke-Davis Co., Morris Plains, NJ) was dissolved in 0.15 M NaCl and stored in small aliquots at –20°C.
in a concentration of 100 U/ml. A23187 (Calbiochem-Behring Corp., La Jolla, CA) was dissolved in dimethylsulfoxide (DMSO) at concentrations of 4, 8, and 12 mM and stored at -20°C. 11C-Arachidonic acid (56.4 mCi/mM) and 5-hydroxy (side chain-2-14C) tryptamine creatine sulfate (50 mCi/mM) were obtained from Amersham, Arlington Heights, IL. Purified thromboxane B2 was a gift from Dr. John Pike (Upjohn Laboratories, Kalamazoo, MI), and thromboxane B2 antibody was kindly donated by Dr. J. Bryan Smith (Cardza Foundation, Thomas Jefferson University, Philadelphia, PA). The antibody against the low affinity platelet factor 4 (LA-PF4) was kindly donated by Dr. Stefan Niewiarowski (Thrombosis Research Center, Temple University, Philadelphia, PA).

Preparation of Biologic Materials

Blood and platelet-rich plasma (PRP) were prepared as described previously.7 The remaining blood was centrifuged at 3,000 g for 30 min at 4°C and the plasma used for coagulation studies. 14C-Arachidonate-labeled PRP was prepared as described previously.6 Gel-filtered platelets (GFP) were prepared according to Lages et al.5 using Ca2+-free Tyrode’s solution, which contained 5 mM glucose and 0.2% human serum albumin.

Platelet Counts, Bleeding Time, and Coagulation Studies

Whole blood platelet counts were determined by both phase-contrast microscopy and electronically ( Coulter Electronics Model Z41, Coulter Electronics, Hialeah, FL). Bleeding time was measured by the template method (Simplate II, General Diagnostics). The prothrombin time, activated partial thromboplastin time (PTT), and factor VIII coagulant activity in plasma were measured by one-stage clotting assays.7

Aggregation and 14C-Serotonin Uptake, Retention, and Secretion in PRP

PRP from patients and normal donors was incubated with 0.5 μM 14C-serotonin, and its incorporation into platelets at 2, 5, 10, and 30 min and retention over 60, 100, 200, and 300 min was determined as described elsewhere.6 The aggregation response of PRP to adenosine diphosphate (ADP) (1–8 μM), epinephrine (1–8 μM), arachidonic acid (0.5 and 1 mM), and ristocetin (1.1 and 1.3 mg/ml) was determined using a Payton dual channel aggregometer. The secretion of the prelabeled 14C-serotonin was determined by stimulating platelets with agonists mentioned above.4

 Liberation of 14C-Arachidonate From Phospholipids in GFP

After gel filtration of 14C-arachidonate-labeled PRP, portions (1 ml) of GFP were incubated with 0.15 M NaCl (control) or thrombin (0.06–5.0 μl/ml) for 5 min at 37°C, without stirring. The samples were extracted with chloroform/methanol, fractionated by thin-layer chromatography, and the amounts of 14C-phospholipids, 14C-arachidonate, and its oxygenation products were determined as outlined elsewhere.6

Thromboxane B2, Production in Spontaneously Clotting Whole Blood

One milliliter of whole blood was allowed to clot spontaneously at 37°C for 30 min. The level of thromboxane B2 was measured by radioimmunoassay8 in the serum and expressed in pmole/108 platelets in whole blood.

Secretion Studies in GFP

Three secretory processes are identified in platelets according to the origin of the secreted substances:11 dense granules (ATP, ADP, serotonin), alpha granules (platelet-specific proteins, fibrinogen), and acid-hydrolase-containing vesicles (acid glycosidases). All three secretory processes can be induced by thrombin and the divalent cation ionophore A23187, and in the present study, these processes were monitored using multiple concentrations of these agonists. The experiments were performed in GFP for several reasons: (1) PRP contains high background (plasma) levels of acid hydrolases, low affinity platelet factor 4 (LA-PF4), and enzymes that break down secreted ATP and ADP, (2) thrombin used to induce secretion would induce clotting in PRP; and (3) A23187 binds strongly to albumin. For thrombin-induced secretion, portions of GFP (1.0 ml) were incubated with 100 μl of 0.15 M NaCl (control) or thrombin (0.15 M NaCl) in various concentrations at 37°C for 2 min without stirring. At the end of the incubation, 50 μl of 0.1 M EDTA (pH 7.4) was added to the incubation mixtures. All the samples, except for one of the two control samples, were centrifuged at 2,000 g at room temperature for 2 min. The secreted ATP plus ADP, LA-PF4, and acid hydrolases were measured in the supernatants of thrombin- and saline-treated GFP, as described below. For the determination of total contents of these substances, the noncentrifuged control sample was extracted. The extent of secretion was calculated as the level of the substance or enzyme activity in the supernatant relative to the total content in the cells.12

To monitor A23187-induced aggregation and secretion, portions (1 ml) of GFP were stirred (900 rpm) at 37°C in the aggregometer with 1 μl of A23187 in various concentrations in DMSO or 1 μl of DMSO (control) and the optical density recorded for 3 min. The cuvette contents were then transferred into precooled tubes that contained one-fifth volume of 0.075 M NaCl/0.05 M EDTA. These mixtures were centrifuged and extracted, and the extent of secretion calculated as described above.

Determination of “Maximally Secretable” Granule Stores

Upon stimulation, platelets secrete less than their total content of a constituent. “Maximally secretable amounts” is defined here as the amount actually secreted from platelets on incubation with 5 U/ml of thrombin at 37°C for 2 min.

Determination of Granule Contents

The concentration of ATP and ADP were determined by a firefly luminescence method.12 The LA-PF4 was measured in samples pretreated with 0.5% Triton X-100 solution by radioimmunoassay, described by Rucinski et al.14 The activities of β-hexosaminidase, β-glucuronidase, and α-mannosidase were determined with appropriate 4-methyl umbelliferone substrates, according to Dangelmaier and Holmsen.15

Statistical Analysis

The data were analyzed by Student’s t test.

RESULTS

Screening Tests for Hemostatic Function

The platelet counts, activated partial thromboplastin time, prothrombin time, and factor VIII activity in plasma were within normal limits in all patients. The bleeding time was 8.5 min in one patient; it was within the normal range (3.0–8.0 min) for the rest of the patients.

From www.bloodjournal.org by guest on November 9, 2017. For personal use only.
Aggregation and Secretion Responses in Platelet-Rich Plasma

Aggregation studies in PRP did not reveal striking abnormalities in patients with ADD. In two patients, the second wave of aggregation was absent with ADP and epinephrine. With arachidonic acid, the extent of aggregation was impaired in one patient. With ristocetin, it was normal in nine of the ten patients tested; in the tenth patient it was absent on one of two occasions studied. On incubation of PRP with $^{14}$C-serotonin, the mean uptake of this biogenic amine by the platelets from the patients and its retention over 5 hr was not different from normals. The secretion of $^{14}$C-serotonin by platelets from ADD patients during aggregation with ADP (4 μM), epinephrine (2 μM), and arachidonic acid (1.0 mM) was 16.9% ± 4% (n = 12, mean ± SE), 18.6% ± 6.0% (n = 11), and 27.3% ± 5.6% (n = 9), respectively. In the normals, the corresponding values were 20.0% ± 2.9% (n = 27), 27.2% ± 3.8% (n = 22), and 23.1% ± 4.3% (n = 16) with ADP, epinephrine, and arachidonic acid, respectively. The differences between the ADD patients and controls were not significant ($p > 0.05$).

Aggregation Response in Gel-Filtered Platelets

Aggregation response to A23187 (4 μM) was absent in 8 of the 12 patients; in 5 patients it was absent at all 3 concentrations of A23187 tested (Fig. 1). As a group, ADD patients had markedly decreased aggregation response ($p < 0.001$) to all concentrations of A23187 used (Fig. 2).

Total Amounts of Platelet Constituents

The total contents of ATP plus ADP and the ATP/ADP ratio in platelets from the patients (6.19 ± 0.43 μmole/10^11 platelets; ratio 1.34 ± 0.08, mean ± SE) were not significantly different from the values in the normals (7.19 ± 0.90 μmole/10^11 platelets; ratio 1.46 ± 0.30). The total levels of LA-PF4 were also the same in the two groups (patients 41.5 ± 5.5, normals 42.5 ± 3.3 μg/10^11 platelets). The total activities of the three acid hydrolases measured were not different in the platelets from the patients as compared to those from normal controls: β-hexosaminidase (patients 5.22 ± 0.63, normals 4.20 ± 1.03 μmole/min/10^11 cells), β-glucuronidase (patients 0.43 ± 0.03, normals 0.60 ± 0.12 μmole/min/10^11 cells), and α-mannosidase (patients 0.42 ± 0.04, controls 0.49 ± 0.12 μmole/min/10^11 cells).

Secretion Response in Gel-Filtered Platelets

The results of studies on secretion of constituents from dense granules, α-granules, and acid hydrolase vesicles by thrombin and A23187 are shown in Figs. 3

---

**Fig. 1.** Tracing of A23187-induced aggregation of platelets from an ADD patient and a normal subject.

**Fig. 2.** Aggregation response to A23187 in GFP. The change (mean ± SEM) in optical density 3 min after addition of A23187 (see Fig. 1). This was calculated in percent of the difference in the optical density between the medium and the unstimulated GFP. The data represent aggregation studies in 12 patients with ADD (hatched bars) and 10 normal individuals (open bars). The concentrations of A23187 (μM) are indicated next to the bars.
DISCUSSION

Our results show that the platelets from patients with ADD and easy bruising have a decreased ability to secrete ATP and ADP from the dense granules, as well as β-hexosaminidase and β-glucuronidase from the acid-hydrolase-containing vesicles. The abnormality was demonstrable in GFP using A23187 and thrombin as the agonists. In addition, the aggregation response in the patients was either absent or markedly impaired upon stimulation of GFP with A23187.

Inherited defects in platelet secretion may occur by several mechanisms. The best characterized mechanism is the storage pool deficiency, where the contents of dense granules are decreased or absent, with or without concomitant deficiency in α-granule substances. In addition, patients with storage pool deficiency have impaired retention of 14C-serotonin when their platelets are labeled with this biogenic amine. The group of patients with ADD described here had normal contents of dense granules, α-granules, and acid-hydrolase-containing vesicles and normal retention of 14C-serotonin, indicating that the secretion defect is not due to a storage pool deficiency. The second major group of platelet secretion disorders results from defects in the arachidonate pathways, such as deficiencies of the cyclooxygenase or thromboxane synthetase enzymes and impairment in the liberation of arachidonate from phospholipids. Patients falling into these categories currently constitute only a few case reports. In the patients described here, the liberation of arachidonic acid and production of thromboxanes was found to be normal. These patients with ADD, therefore, belong to a new category of platelet secretion disorders that is characterized by normal storage pools and normal arachidonate pathways.

The impairment in platelet aggregation and secretion in ADD patients was demonstrable in GFP upon stimulation with A23187 and thrombin, but not in PRP using the usual agonists, such as ADP and epinephrine. It may be that the failure of aggregation in GFP is more specific for A23187 and thrombin as the agonists. In addition, the aggregation response in the patients was either absent or markedly impaired upon stimulation of GFP with A23187.

Studies on Arachidonate Metabolism

There were no significant differences in 14C-arachidonate liberation observed between normal subjects and patients at all concentrations (0.06, 0.1, 0.5, 1.0, and 5.0 U/ml) of thrombin tested (results not shown). Furthermore, the mean thromboxane-B2 production in clotting whole blood of the 12 ADD patients (180.6 ± 27.5 pmole/10⁸ platelets) was not statistically different from that of 10 normal subjects (124.5 ± 16.4 pmole/10⁸ platelets), suggesting that the secretion defect is not due to impaired arachidonate liberation or thromboxane formation.

Fig. 3. (A) Dense granule (ATP + ADP) secretion. (B) α-Granule (LA-PF4) secretion. Secretion of dense granule (ATP plus ADP) and α-granule (low affinity platelet factor 4, LA-PF4) contents on stimulation with A23187 and thrombin. The asterisks represent the maximally secretable amounts, defined as the secretion induced by a high thrombin concentration (5 U/ml). The mean and SEM are shown for 10 normals (open bars) and 12 patients with ADD (hatched bars).
tion could be greater in ADD platelets than in normal platelets, thereby providing a clearer distinction between the two groups. Irrespective of the mechanisms, platelets from the patients can be shown to have an impairment in their secretory mechanism that is revealed by an agonist that is independent of close cell contact (thrombin) and an agent that is dependent on such cell contact, A23187 and which is not due to deficiencies in the granule contents or in the arachidonate pathway. As the first steps in the stimulus–response coupling in patients are believed to be common for aggregation and secretion, platelets from the ADD patients may have impairments in the early events, such as liberation of Ca\(^{2+}\) into the cytoplasm.

Interestingly, \(\alpha\)-granule secretion was not significantly impaired in the ADD platelets. Our findings suggest that \(\alpha\)-granule secretion may be governed by mechanisms distinguishable from those mediating dense granule secretion. In addition, while secretion of \(\beta\)-glucuronidase and \(\beta\)-hexosaminidase was abnormal, secretion of \(\alpha\)-mannosidase was normal upon stimulation of the patients' platelets with A23187. These observations suggest that there may be differences in the mechanisms governing the secretion of acid hydrolases and that acid hydrolase secretion in platelets is heterogeneous.

As the majority of the adult female ADD patients seen by us (P.S.M.) have had symptoms of easy bruising, and because of the similar characteristics of the platelet defect demonstrated by us in these patients, it is tempting to speculate that there may be a relationship between platelet secretion and ADD.
Platelets have several features in common with neurons. They have the ability to accumulate, store, and secrete biogenic amines, and it has been previously proposed that these cells may serve as a model for monoamine-containing neurons. Consequently, characteristics of platelets from patients with various neuropsychiatric diseases have been studied by several investigators. In these studies, three platelet parameters have been considered: uptake and efflux of amine (e.g., 5-hydroxytryptamine), storage of amine, and the activity of monoamine oxidase. In contrast, the mechanism of secretion, a cellular function that plays a pivotal role in neuronal transmission, has (for unknown reasons) not been previously studied in platelets of patients with neuropsychiatric disorders. Therefore, our examination of the secretion mechanisms in platelets from ADD patients and demonstration of a defect in it, refocuses attention on the concept of platelets as a model for neuronal transmission and highlights another facet—secretion—which could be specifically investigated in future studies in patients with psychiatric disorders.

To our knowledge, the impaired platelet dense granule secretion in the ADD patients is the first demonstration of a cellular or tissue disorder in this functional psychiatric illness. There have been scattered reports regarding biochemical disturbances that may be related to ADD in children. Wender advanced the hypothesis that “minimal brain dysfunction” (ADD) may be caused by a disturbance in monoamine metabolism. Errors in the central nervous system, which are partially corrected by methylphenidate, have been suggested as a pathophysiologic cause of childhood ADD. The changes in the levels of certain monoamine metabolites in cerebrospinal fluid and urine from hyperkinetic children in response to d-amphetamine, which partially normalizes the adverse behaviour as well, also support the “disturbed monoamine metabolism” hypothesis. However, none of these studies depicts a clear biochemical or cellular defect in ADD. Our studies on platelets from 12 patients with ADD and easy bruising show a defect in the secretion from dense granules—the amine-containing granules—and acid hydrolases, but not the α-granules—the protein-storing granules. Additional studies from our laboratory suggest that impairment in acid hydrolase secretion is most likely secondary to the impaired dense granule secretion (Lages B, Holmsen H, unpublished observations.) Thus, the primary defect in the platelets from patients with ADD may be impaired secretion from the amine-containing dense granules, and may possibly be due to an impairment in Ca2+ mobilization. Because of the common features between neurons and platelets in handling biogenic amines, these observations permit one to speculate that mechanisms for the neuronal secretion from amine-containing granules may be impaired and may constitute the biochemical defect in ADD. If these observations are confirmed, the easily demonstrable platelet defect in ADD would provide new avenues for studies on patients with ADD. Finally, the study of platelet secretion in disorders such as ADD may have a heuristic value in conceptualizing the basic defects in neuropsychiatric disorders.

ACKNOWLEDGMENT

We are grateful to Drs. Sol Sherry and H. James Day for the support provided for this study. We thank Terry Cruice and Mindy Noble for excellent secretarial assistance, and Carol Dangelmaier for her assistance at various stages of the project.

REFERENCES

Platelet secretion defect in patients with the attention deficit disorder and easy bruising

K Koike, AK Rao, H Holmsen and PS Mueller