Platelet Factor 4 in Platelet Disorders—Storage Location and the Requirement of Endogenous ADP for Its Release[1] (36951)

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When platelets are stimulated by collagen or epinephrine, among other substances, adenosine diphosphate (ADP), present in a nonmetabolic or storage pool of adenine nucleotides, is released and results in platelet aggregation (1). Massini and Lüscher (2) have recently suggested that this storage pool of ADP, in addition to being released, may also play a role in the release mechanism itself. We therefore thought that it would be useful to study some aspects of this mechanism in patients whose platelets are deficient in the storage pool ADP (storage pool disease) (3, 4). We studied the release of platelet factor 4 (PF-4) (5, 6), the heparin-neutralizing factor (7, 8). PF-4 activity has been demonstrated in isolated platelet granules (9), but it is not clear whether these are the electron-dense granules which contain the storage pool of adenine nucleotides or the less electron-dense lysosomal granules (α-granules) (1, 10, 11). Therefore, we thought that a study of PF-4 activity in patients with storage pool deficiencies and other platelet disorders would be useful in this regard also.

Materials and Methods. 1. *Subjects.* a. *Storage pool disease.* Six patients whose platelets are deficient in the storage pool of ADP and serotonin were studied (3, 4, 12, 13). Three patients (DC, SC and RC) are members of a family with a unique platelet lipid abnormality (family C) (14), while the other 3 patients (SN, EP and LG) are unrelated (13).

b. *Thrombasthenia.* Two patients (LW and MC) with classical Glanzmann's thrombasthenia have been the subjects of previous reports (16, 17). Their platelets are not aggregated by any concentration of ADP.

c. *Aspirin-induced defect.* Two normal subjects were studied before and 1 day after ingesting 1.2 g of aspirin.

d. *"Aspirin-like" disorder.* The type of platelet aggregation defects in patient FA, as well as in normal subjects who ingest aspirin, are similar to those in storage pool disease (13). These "aspirin type" of defects, however, are owing to an impairment in the release of storage pool ADP, which is present in normal amounts (4).

e. *Normal subjects.* Nine normal subjects who had not ingested any drugs for at least 1 wk were also studied.

2. *Methods.* a. *Platelet-rich plasma (PRP).* Blood was mixed (9:1) with 3.2% sodium citrate and centrifuged for 3 min at 1500*g* and 20° to obtain PRP, as previously described (13). An aliquot of this PRP was centrifuged for 30 min at 3200*g* and 20° to obtain platelet-poor plasma (PPP). The platelet count in the PRP was adjusted with autologous PPP to 200,000/mm^3.

b. *Platelet aggregation and PF-4 release.* PRP (2.5 ml) was stirred at 37° by means of a Teflon-coated magnet, rotating at 1000 rpm, in the cuvette of a Peyton dual channel aggregometer (Buffalo, NY). Aggregating agents were added in a volume of 0.1 ml and consisted of an undiluted suspension of washed connective tissue (17), a 1:5 dilution of this suspension and epinephrine in a final concentration of 5 μM. Platelet aggregation was recorded on a Riken-Denshi recorder and the transmittance values, compared with a PPP blank, were converted to optical density values. Platelet aggregation was expressed as percentage change of optical density (13). Six minutes after adding the connective tissue

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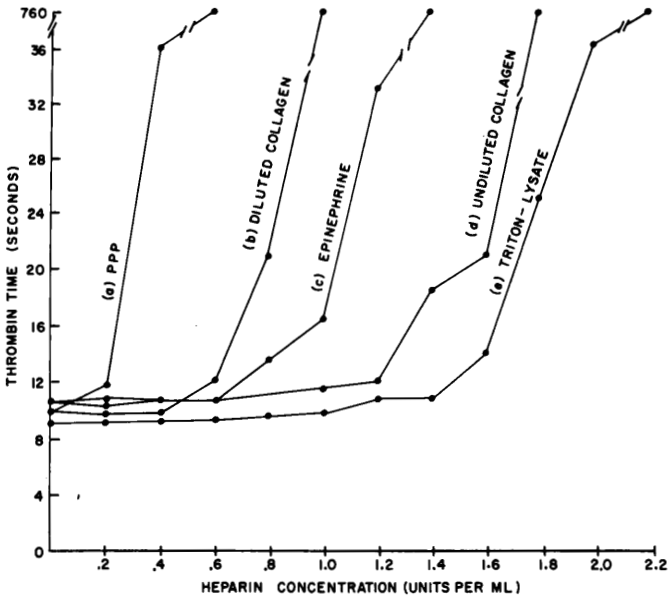

FIG. 1. Platelet factor 4 assay. Normal PRP was (a) centrifuged to obtain PPP; (b, c, d) stirred with collagen or epinephrine and then centrifuged; (e) lysed with Triton-X. Clotting mixture contained 0.2 ml of test substance + 0.05 ml of heparin solution (conc indicated) + 0.1 ml of thrombin, 10 units/ml.

suspension (hereafter referred to as collagen) and 10 min after adding the epinephrine, the contents of the cuvette were centrifuged for 5 min at 1000*g* in a Clay-Adams serofuge to remove the large platelet aggregates. The supernatant was then centrifuged again for 45 min at 17,000*g* and 4° and the supernatant plasma was removed. An aliquot of PRP was centrifuged in a similar manner to obtain platelet-poor plasma and another aliquot was treated with 1/40 volume of a 20% solution of Triton X-100 in normal saline for 5 min at 37°, which resulted in lysis of the platelets. All specimens were stored at —60° for no longer than 3 wk.

c. *PF-4 assay.* The stored specimens indicated in (b) were thawed and PF-4 was measured as heparin neutralizing activity by the thrombin time method of Harada and Zucker (18). In this method, graded amounts of heparin are added to the specimens and the thrombin clotting time is determined. The test was performed by determining the clotting time on a mixture containing 0.2 ml of the test specimen + 0.05 heparin solution + 0.1 ml of a thrombin solution containing 500 units of thrombin/ml of 50% glycerol–saline, stored at —20°. Sodium heparin (Upjohn), 5000 units/ml of normal saline, was diluted to concentrations ranging from 0.2 to 3.0 units/ml of saline. With increasing concentrations of heparin, the thrombin clotting time remains constant until the amount of heparin added exceeds the heparin-neutralizing capacity of the test substance. With subsequent increments of heparin, the clotting time increases and then abruptly becomes infinite and from these values a curve relating the thrombin clotting time to the heparin concentration can be drawn. (Fig. 1).

d. *Calculation of PF-4 activity.* The concentration of the heparin solution which gave a thrombin clotting time of 20 sec was determined from the curve and the number of heparin units contained in 0.05 ml of this solution was defined as the heparin-neutralizing or PF-4 activity of the test material. Since a small amount of heparin-neutralizing activity was present in PPP (Fig. 1), this activity was subtracted from the values obtained for the other specimens to obtain the amount of PF-4 attributable to the platelets (Triton-treated specimen) or to the activity released from the platelets by collagen or

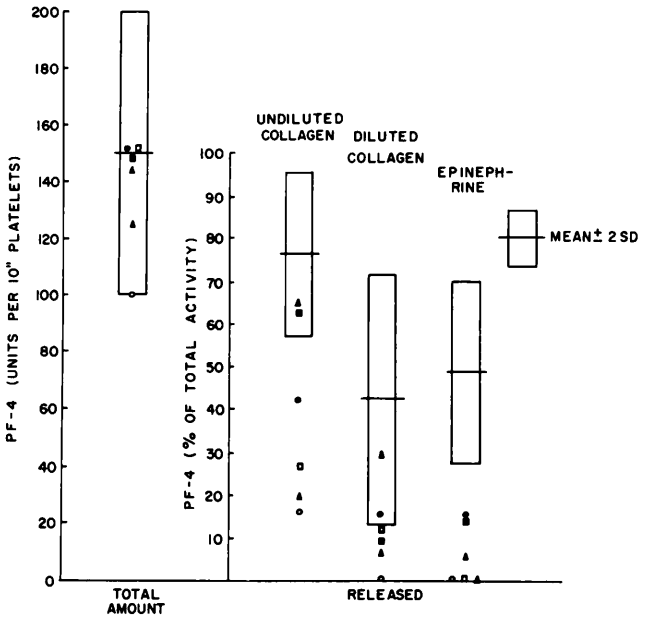

FIG. 2. Platelet factor 4 activity and release in patients with storage pool disease: (a) *family C,* (○) DC, (□) SC, (△) RC; (b) other, unrelated patients (●) SN, (■) EP, (▲) LG. Normal values are indicated by the bars (mean ± 2 SD).

epinephrine. Results were expressed either as units of heparin neutralized per 10^{11} platelets (total activity) or as the percentage of this activity which was released.

Results. 1. *Normal subjects* (Fig. 1). In 9 normal subjects, the total amount of PF-4 activity in the platelets was 149 ± 26 (SD) units/10^{11} platelets. With the undiluted collagen suspension, 77 ± 11 (SD)% of the total PF-4 activity was released from the platelets and recovered in the supernatant. With the diluted collagen suspension, 43 ± 17% was released, while with epinephrine, 49 ± 12% was released.

2. *Storage pool disease* (Fig. 2). The total amount of PF-4 activity in the platelets of these patients was within the range of normal values. This was even the case in the 3 members of family C whose platelet volumes are, on an average, 70% that of normal platelets (12). In contrast to the normal content of PF-4 in storage pool disease, the percentage of this activity which was released from the platelets was decreased. For the group as a whole, this abnormality was most strikingly demonstrated by the values obtained for epinephrine-induced PF-4 release, which were zero in 3 patients and significantly decreased in the 3 others. A pattern of decreased PF-4 release was also obtained with the diluted collagen preparation. The defect in PF-4 release was most striking in the 3 members of family C, in whom markedly decreased values were obtained even when undiluted collagen was used. In 2 of the other 3 unrelated patients, undiluted collagen released a normal amount of PF-4.

3. *Thrombasthenia* (Fig. 3). Thrombasthenic platelets contained a normal amount of PF-4 activity. However, little or none of this activity was released by epinephrine or diluted collagen. By contrast, undiluted collagen released a normal amount (60%) of PF-4 activity from the platelets of one patient (LW), while the value of 45% released from the platelets of MC was only slightly outside the range of normal values.

4. *Aspirin* (Fig. 3). The total amount of PF-4 activity was unaffected by aspirin ingestion. The release of PF-4 by both undiluted and diluted collagen and by epinephrine was significantly decreased, however. A similar pattern was obtained in the patient with an "aspirin-like" defect except that the percentage of the PF-4 activity released by undiluted collagen was normal.

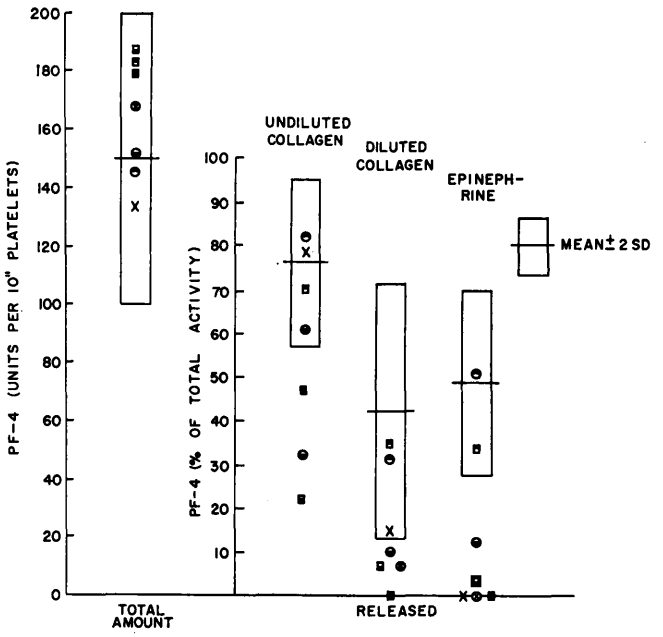

FIG. 3. Platelet factor 4 activity and release in (a) thrombasthenia (⊗) LW, (⊠) MC; (b) "aspirin-like" disorder, (×) FA; (c) normal subjects before (● ■) and after (● ■) aspirin ingestion. Normal values are indicated by the bars (mean ± 2 SD).

5. *Correlation between PF-4 release and platelet aggregation.* The results obtained with the undiluted collagen preparations are shown in Fig. 4. If the patients with thrombasthenia are excluded from the analysis, there appears to be some correlation between the degree of aggregation and the percent of PF-4 released from the platelets of the other 9 subjects ($r = .769$, $p < .05$). In the 2 thrombasthenic patients, however, relatively large amounts of PF-4 were released despite a markedly diminished amount of aggregation (surprisingly some aggregation did occur with this undiluted collagen preparation).

Discussion. There is some evidence which suggests that PF-4 may be stored in the platelet-dense granules. For example, it is released early in the course of the release reaction in parallel with ADP (19) and serotonin (18) which are known to be stored in the dense granules (1, 10, 11). The lysosomal enzymes of the α-granules, by contrast, are released more slowly (11). In addition, PF-4 can be released by epinephrine, a weak release-inducer which releases ADP and serotonin but not β-glucuronidase (20), a constituent of the α-granules (1, 11). In the present study, we measured PF-4 activity in patients with storage pool disease whose platelets contain fewer dense granules than normal subjects (21) and which are deficient in at least 3 of the constituents of these storage granules (ADP, ATP and serotonin) (3, 4). Therefore, the finding that the PF-4 content of their platelets was *normal* suggests that PF-4 may not be stored in the dense granules. Of course, it is also possible that PF-4 and the other storage substances *are* normally present in the same dense granules and that the normal amount of PF-4 in patients with storage pool disease is owing to an increase in the amount of soluble PF-4 (9) or to a selective type of abnormality in the dense granules.

Although the total amount of PF-4 in storage pool disease was normal, the percentage of the total activity which was released from their platelets by diluted collagen and by epinephrine was significantly less than in normal subjects. In 4 of the 6 patients, even undiluted collagen did not release a normal amount of activity. We have also found that the release of platelet-bound ^{14}C-serotonin is decreased in storage pool

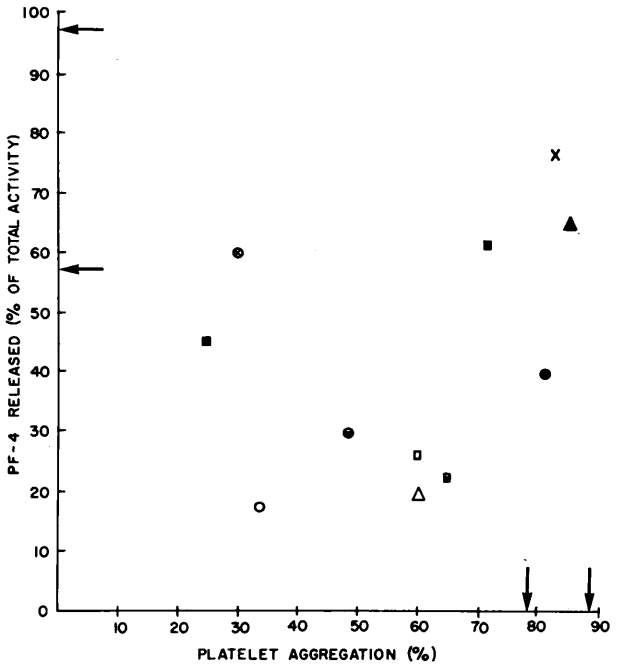

FIG. 4. Relationship between platelet factor 4 release and platelet aggregation. The results obtained with undiluted collagen are shown. Symbols for the thrombasthenic (⊗ ⊠) and other patients are the same as in Figs. 2 and 3. Arrows denote the limits of the values in normal subjects. Excluding the 2 thrombasthenic patients, ($r = +.769$, $p < .05$).

disease (22). Although the defects in the release reaction and the deficiency of the storage pool in these patients may not be causally related, these findings nevertheless raise the possibility that the storage pool of ADP may play an important role in the release mechanism. A similar conclusion was reached by Massini and Lüscher (2), who showed that endogenous ADP may be necessary for the release reaction induced by cationic polymers.

The nature of the requirement of storage pool ADP for the release reaction requires further clarification. It could be related to the role of endogenous ADP in producing platelet aggregation, which might then induce the release reaction by a "propinquity" effect (23). There appeared to be a good correlation between the decreased PF-4 release and decreased aggregation induced by epinephrine, as in previous studies (18, 24, 25). This was also the case with undiluted collagen in 9 of the 11 subjects. However, in the 2 thrombasthenic patients, strong collagen induced the release of a relatively large percent of the PF-4 activity despite the fact that only a very small amount of platelet aggregation occurred, and similar findings in thrombasthenia were also obtained by Kubisz *et al.* (24) and by Harada and Zucker (18). Therefore, platelet aggregation does not appear to be necessary for the release of PF-4 and the role of storage pool ADP in the release reaction must be owing to some property other than its ability to aggregate platelets. The nature of this role requires further clarification.

Summary. Platelet factor 4 was present in normal amounts in patients whose platelets are deficient in the storage pool of adenine nucleotides and in the electron-dense granules in which these substances are stored. These findings suggest that PF-4 may be stored in other granules. The release of PF-4 by collagen and epinephrine, however, was decreased in these patients and these findings suggest that the storage pool of ADP may, in addition to being released, play a role in the release mechanism. The findings in patients with thrombasthenia indicate that this is not related to the ability of storage

pool ADP to aggregate platelets.

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