Platelet-Bound and Soluble Platelet Factor 4: Effects of Aggregating Agents, of Aggregation, and of Aspirin[1] (34958)

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(Introduced by M. B. Zucker)
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Platelets can inactivate heparin, an attribute called Platelet Factor 4 or heparin neutralizing activity (HNA). There are two recent apparently conflicting findings. If adenosine diphosphate (ADP) or epinephrine is added to stirred human platelet-rich plasma (PRP) at 37° no HNA is evident for about 2 min; thereafter considerable activity is released (1). Another report (2) claims that immediately after the addition of ADP to *stationary* PRP some HNA is detected and that epinephrine is inactive. Furthermore, aspirin is said to inhibit the development of HNA (2, 3). This report is an attempt to reconcile these observations and to explore the situation further.

Methods. Fresh human citrated platelet-rich plasma (PRP), 3.0 ml, was stirred at 37° in the cuvette of an aggregometer, and 1.0 ml of an aggregating agent was added; a tracing recorded the degree of aggregation against time (4). At appropriate intervals, judged by the degree of aggregation, samples of the PRP were removed and tested for HNA by adding a known amount of heparin and then measuring the thrombin clotting time (2). For convenience the units of heparin neutralized by 10 ml of PRP were recorded. This test measures the total HNA. Exactly similar proportions of PRP and the aggregating agent were also mixed in small tubes and after the same intervals of *stationary* incubation, the HNA activity was determined by adding heparin and thrombin directly to these tubes. These two treatments are called Stirred (when aggregation occurs) and Not Stirred (when no significant aggregation occurs). After these two types of treatment, and at the same time intervals, yet other aliquots of PRP were centrifuged at 688*g* for 1 min, and the supernatant was then tested at once for HNA. Separate experiments showed that any HNA activity in this supernatant did not decrease after additional centrifugation at 260,000*g* for 30 min; such activity is called soluble HNA. 688*g* spins down most of the platelets; if HNA is removed from the supernatant by this procedure, then this activity is called platelet-bound HNA.

Results. Figures 1–4 portray typical tracings from the aggregometer and indicate when samples were removed for testing HNA. The extent of the downward deflection indicates the degree of aggregation. Table I indicates the mean amount of HNA produced in 2–10 separate experiments.

When reversible aggregation was induced in stirred PRP by a small amount of ADP (Fig. 1), virtually no HNA was demonstrable. Unstirred, the PRP developed HNA at once and since none was in the supernatant, *i.e.*, soluble, evidently all the HNA was platelet-bound.

Figure 2 is a record of ADP-induced aggregation with a second wave indicating release. In the stirred sample much HNA developed after 3 min, and since none was lost during centrifugation, it must all have been soluble. When the PRP was not stirred, some HNA developed at once and persisted unchanged; evidently it was all platelet-bound since none was soluble.

Aliquots of PRP were also incubated with aspirin ($M \times 10^{-3}$) for 10 min, and then similarly tested. There was no double wave of aggregation so presumably no release oc-

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TABLE I. The Development of Total and Soluble HNA and of Release in PRP After Various Forms of Treatment.[a]

	Sample identification (time of test)					
	A	B	C	D	E	F
Normal PRP + ADP.						
Reversible aggregation (Fig. 1)						
Stirred						
Total HNA	0	1	1	0	0	0
Soluble HNA	0	0	0	0	0	0
Release occurs	0	0	0	0	0	0
Not stirred						
Total HNA	0	4	4	4	5	6
Soluble HNA	0	0	0	0	0	0
Normal PRP + ADP.						
Double wave aggregation (Fig. 2)						
Stirred						
Total HNA	0	0	0	1	15	21
Soluble HNA	0			0	14	25
Release occurs	0	0	0	+	++	++
Not stirred						
Total HNA	0	4	4	4	5	6
Soluble HNA	0	0	0	0	0	0
Normal PRP + epinephrine. (Fig. 3)						
Stirred						
Total HNA	0	1	1	8	17	19
Soluble HNA	0			7		17
Release occurs	0	0	+	++	++	++
Not stirred						
Total HNA	0	1	1	1	1	1
Soluble HNA	0	1	0			1
Normal PRP + Collagen. (Fig. 4)						
Stirred						
Total HNA	0	1	7	19	27	27
Soluble HNA	0		6			29
Release occurs	0	0	+	++	++	++
Not stirred						
Total HNA	0	3	4	3	5	5
Soluble HNA	0		1			5

[a] PRP was tested for HNA after the treatment indicated and at the times after the addition of the aggregating agent indicated on Figs. 1, 2, 3, 4, by A, B, etc. The values are the average units of heparin neutralized by 10 ml of PRP. Release is reported to have occurred when ADP or epinephrine caused a second wave of aggregation and when collagen induced aggregation. Full release, ++; partial or early release, +; no release, 0.

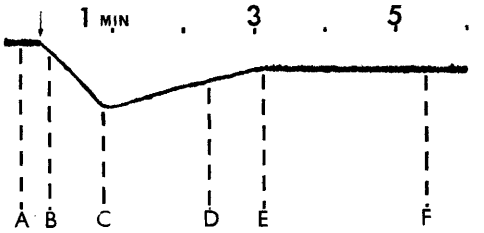

FIG. 1. At the arrow ADP, final concentration 2.5 $\times$ 10^{-6} M is added to stirred PRP; aggregation is followed by disaggregation. Samples of PRP were removed for testing at the times indicated, *viz*, at A (before the addition) and at B, C, D, E, and F.

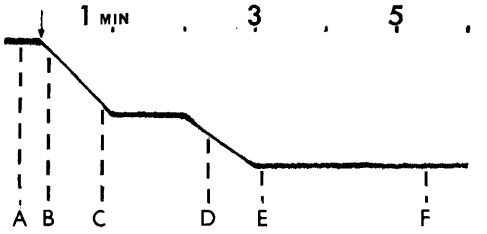

FIG. 2. At the arrow ADP, final concentration 5 $\times$ 10^{-6} M is added to stirred PRP. The double wave of aggregation indicates that release begins during the third minute.

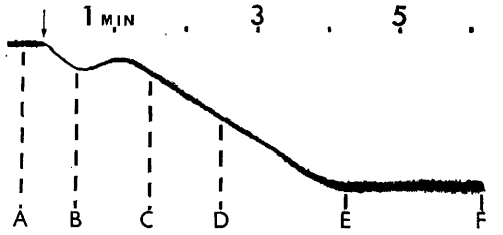

FIG. 3. At the arrow, epinephrine 5 $\times$ 10^{-7} M was added to stirred PRP. A double wave indicates release.

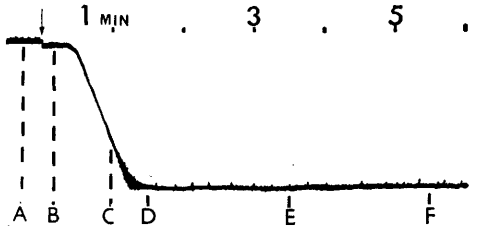

FIG. 4. At the arrow, a suspension of collagen (a crude tendon extract) was added to PRP.

curred. When this plasma was stirred, a small amount of platelet-bound HNA (4 units) developed, but only after 6 min. Unstirred,

negligible quantities of HNA (1 unit) developed.

Epinephrine added to stirred PRP caused a double wave of aggregation (Fig. 3) and a large amount of HNA, all soluble, was demonstrable, but only after 3–5 min. The addition of epinephrine to unstirred PRP resulted in very little soluble or platelet-bound HNA. When epinephrine was added to aspirin-treated PRP and stirred, only the first wave of aggregation occurred; virtually no HNA developed either in the stirred or the unstirred samples of PRP.

When a suspension of collagen (a crude tendon extract) was added to stirred PRP, aggregation was complete after about 2 min and much soluble HNA was found (Fig. 4). Collagen added to unstirred PRP caused an immediate exposure of a small amount of HNA (4 units) and initially most of this was platelet-bound; some soluble activity developed after 5.5 min. When such plasma was pretreated with aspirin, the collagen-induced aggregation was inhibited but ultimately a small amount of soluble HNA (4 units) was exposed. When aspirin-treated PRP was not stirred no activity developed at all.

The data in Table I appears to indicate that the amount of soluble HNA released differed when aggregation was induced by ADP, epinephrine, and collagen; nevertheless, when aliquots of one sample of PRP were exposed to these reagents an equal amount of HNA was exposed. Different plasmas treated identically developed different amounts of HNA, causing the differences noted in Table I.

Discussion and Conclusions. These studies confirm previous work (1–3). Some platelet-bound HNA develops immediately after the addition of ADP and collagen (2) to unstirred PRP; presumably a change in the platelet surface has occurred. However, no platelet-bound HNA is found when the platelets are stirred and aggregate. Presumably the platelet-bound HNA develops and then disappears. This disappearance may be due to the decrease in total surface area when platelets aggregate; for example, a clump of 100 platelets has about one sixth of the surface area of 100 separate platelets. Alternatively, the sites of HNA may be related to the development of stickiness and be involved when the platelets stick together.

In agreement with previous work (1), the release of soluble HNA is now shown to occur only when the general release reaction occurs. In the presence of aspirin (5, 6) and in the absence of stirring (7) the release reaction does not occur, and no soluble HNA can be demonstrated (3). Thus the release of soluble HNA may be part of the general release reaction. Aspirin also inhibits the development of platelet-bound HNA. The latter appears to be a separate and independent effect but clearly the two types of aspirin-induced inhibition of HNA may be related and may involve the platelet membrane (2).

It has been suggested (7) that an aggregating agent does not itself cause the release of ADP. The propinquity of platelets to a surface like collagen or to each other, for example during aggregation or centrifugation, seems essential for the release reaction as a whole. Propinquity is now also shown to be essential for the release of soluble HNA. Theoretically the difference between reactions in stirred and unstirred PRP might be due, not to propinquity but to turbulence delivering more reagent to the platelets, while unstirred platelets gain reagent only by diffusion. This possibility seems unlikely but cannot be disproved.

The present and other findings (2) show that no platelet-bound HNA is produced by the addition of epinephrine to unstirred and unaggregated platelets. This, then, is another difference between the effects of ADP and epinephrine on platelets (7). The soluble form of HNA is demonstrable only after epinephrine-induced aggregation has occurred with a double wave indicating release. These findings apparently differ from those of Youssef and Barkan (3); but they used "gentle agitation for 2 min" which may induce some degree of release.

These findings also indicate the molecules responsible for HNA can exist in three forms. They may be (a) hidden and inactive in the platelet surface, (b) active at the platelet

surface (platelet-bound HNA), or (c) in a soluble form after the release reaction. The same or another form is found in serum (8). So far, there is no evidence to indicate whether these three forms are the same or different molecules.

Summary. If ADP is added to unstirred PRP, no aggregation occurs and platelet-bound heparin neutralizing activity (HNA) develops immediately; aspirin prevents the development of this HNA. ADP added to stirred PRP causes aggregation and no platelet-bound HNA is found. It is confirmed that if the release reaction occurs then a soluble form of HNA develops; pretreatment of the PRP with aspirin inhibits both the release reaction and the release of HNA. Adding epinephrine to PRP is not followed by demonstrable platelet-bound HNA, but soluble HNA is found if aggregation and release occur. The development of platelet-bound HNA presumably indicates a profound change in the platelet membrane.

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