A Technique for Estimation of Platelet–Collagen Adhesion[1] (34362)

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In the formation of a hemostatic plug, early events are thought to include platelet build-up on exposed collagen (1). The formation of a platelet mass on collagen fragments or fibers as studied *in vitro* occurs in two steps: Adhesion of the platelets is followed by aggregation, with increasing numbers of platelets sticking to those already adhering. Platelet adhesion to collagen, and aggregation, appear to be basically different reactions (2). One notable difference is that aggregation requires the presence of divalent cation, whereas adhesion occurs even in the presence of strong chelation.

Interaction between platelets and connective tissue has been extensively studied by optical density (OD) methods in citrated platelet-rich plasma (PRP) (3). However, in this system adhesion is not separately detected because the effects of platelet aggregation produce the major OD changes. Recently, Hirsh *et al.* (4) studied selective adhesion in EDTA–PRP by following optical density and platelets counts when a spin-bar wrapped with collagen was used as a platelet trap. Although the amount of collagen was specified, no attempt was made to standardize surface area. In the present method surface area was controlled by using connective tissue fragments of fairly constant sedimentation properties and in a measured amount; optical density was monitored as in the earlier report.

Materials and Methods. Suspensions of connective tissue fragments rich in collagen were prepared from human subcutaneous fat by a modification of the method described by Zucker and Borrelli (5, 6). A highly concentrated final suspension was used which showed the following characteristics: Diluted 1:40 it gave an optical density (OD) of 0.15 in a Beckman DB spectrophotometer at 625 mμ against a distilled water blank; this was the same OD given by Dow polystyrene latex particles, lot no. LS 1117-B, particle size 0.79 μ, and at a concentration of 34,000/mm^3; and the preparation had a titer of 1:10,000 in its ability to produce maximal aggregation of platelets in citrated PRP. In addition, a more dilute preparation was made for certain experiments, as previously described (7). Blood was collected from healthy laboratory personnel on no medication, and was anticoagulated with 0.1 vol of 1% EDTA in isotonic saline, 10% EDTA in distilled water, or 3.8% sodium citrate. The PRP was collected from blood specimens centrifuged at 900 rpm for 15 min in an International Clinical centrifuge. The OD measurements on PRP were performed in an "aggregometer" (Chrono-Log Corp., Broomall, Pa.), connected to a Heath EU-20V output recorder. Experiments were performed at least in duplicate; all procedures were completed at room temperature except that "aggregometer" studies were at 37°. Tests were accomplished within 2 hr of blood collection.

Adhesion of platelets to connective tissue fragments was estimated as follows: EDTA-anticoagulated PRP was used. First the "aggregometer" was calibrated. The recorder pen was set at about 65 units, and 0.05 ml of concentrated connective tissue fragment suspension were added to 1 ml of EDTA–PRP. A progressive fall in OD occurred, which was allowed to continue until a plateau was reached. The recorder was then adjusted so that this connective tissue fragment-treated PRP was set at "zero," and

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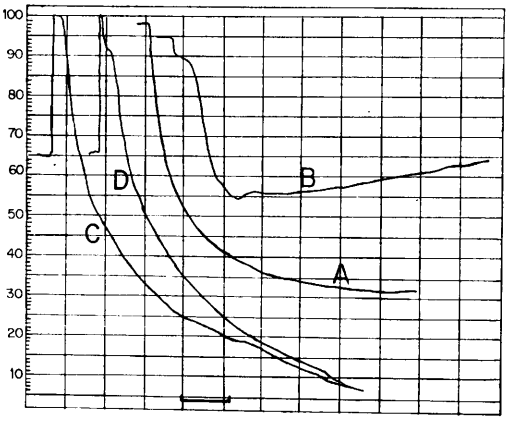

FIG. 1. Optical density tracings following addition of connective tissue suspensions to platelet rich plasma (PRP). (A) Citrated PRP, dilute connective tissue. (B) Citrated PRP with aspirin, dilute connective tissue. (C) EDTA–PRP, concentrated connective tissue. (D) EDTA–PRP with aspirin, concentrated connective tissue. Time line at bottom of graph represents 1 min.

PRP was set at 65 units. In the actual test, 0.05 ml of suspension were added to 1 ml of PRP at the obtained instrument sensitivity, again with PRP set at 65 units. In studies performed with citrated PRP, dilute suspensions were used, and our previous method was employed (6).

Results. Following the addition of concentrated connective tissue fragment suspension to EDTA–PRP, a sharp rise in OD was seen, representing addition of the highly turbid suspension. This was followed by a progressive fall in OD (Fig. 1), which was associated with lining up of platelets on connective tissue fibers. Identical findings were obtained with blood collected in 1 or 10% EDTA, and examination of "aggregometer" specimens by phase microscopy showed selective platelet adhesion to the fibers as illustrated in Fig. 2. There also appeared to be clumps of platelets, but these evidently represented platelets adhering in large numbers to more complex connective tissue formation, or mixed clumps of multiple fibers and platelets mutually interacting.

The preparations showed an additional finding of interest: Virtually all the platelets were seen to be participating in the adhesion reaction, and when the amount of connective tissue preparation was increased slightly almost no free platelets could be seen. These observations contrast with those of Hirsh *et al.* (4), who reported that young platelets selectively adhere.

The application of this test to the question of aspirin action upon platelet–collagen interaction was explored. Platelets exposed to aspirin respond with diminished aggregation following addition of collagen (7). Whether the initial adhesion reaction is depressed, or whether the defect represents an effect upon subsequent release of or response to adenosine diphosphate (ADP) has not been established. Aspirin-treated platelets were obtained by either *in vitro* or *in vivo* drug exposure. *In vitro*, 0.1 vol of 10^{-3} *M* aspirin in isotonic saline, neutralized to pH7 with 0.1 *N* NaOH, was added to EDTA or citrated PRP. The mixture was allowed to stand at room temperature for 15 min before testing. Similarly treated control specimens had isotonic saline substituted for aspirin. Subjects were given 1200 mg of aspirin by mouth; blood specimens were obtained just prior to and 3 hr after drug ingestion. As shown in Fig. 1, the response of citrated PRP to dilute connective tissue fragment suspensions showed definite depression of platelet aggregation in the aspirin-treated platelets, as noted in earlier studies (8). However, in EDTA–PRP exposed to concentrated suspensions, no difference in the adhesion reaction was noted between aspirin-treated and control platelets. Identical results were obtained with *in vivo* or *in vitro* aspirin action.

Discussion. Although the technique described herein is thought to be specific for the platelet–collagen adhesion reaction, two considerations deserve comment. The first concerns the nature of the reactive material in the admittedly crude preparation of connective tissue fragments. Strong evidence favors this to be collagen, since activity is lost with collagenase treatment (5), and electron microscopy of this preparation shows the material to be composed largely of recognizable collagen fibers with typical cross-striation (Baumgartner, H. R., unpublished observa-

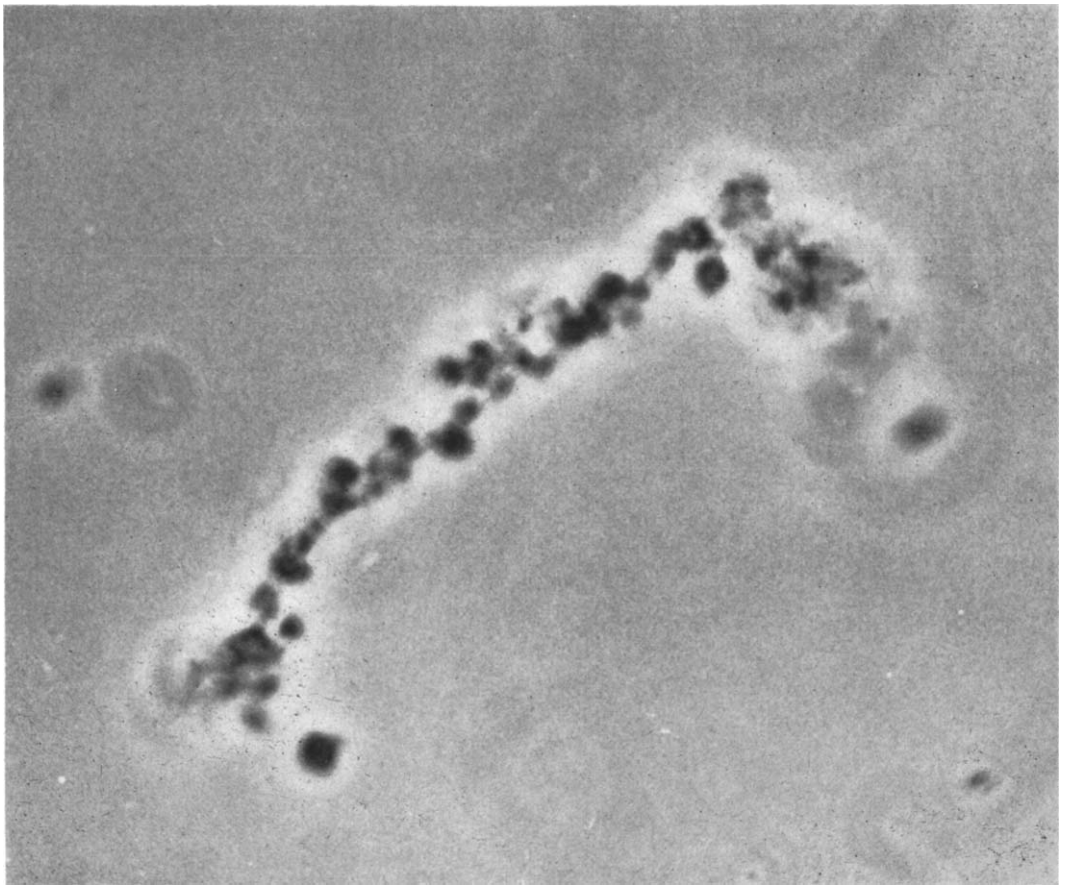

FIG. 2. Phase photomicrograph of specimen from EDTA–PRP reacted with connective tissue fragments. A collagen fiber is shown with adhering platelets; magnification 800×.

tions). The second consideration is whether adhesion alone is being measured, or whether aggregation may occur as well. The latter possibility appears to be unlikely, since platelet aggregation requires divalent cation (8). Not only did the fall in OD occur at EDTA levels which prevented aggregation by smaller concentrations of connective tissue fragments and by high concentrations of adenosine diphosphate but increasing the amount of EDTA tenfold over the usual test amounts failed to influence the results.

The present method is useful for estimating platelet–collagen adhesion in EDTA–PRP, but similar success has not been obtained for washed platelets. For reasons unclear at present, OD readings in plasma-free systems have not well differentiated adhering from free platelets even in the presence of significant adhesion, as verified by phase microscopy.

Several immediate applications of the adhesion technique are suggested. The first is the distinction between adhesion and release in several platelet abnormalities, including acquired or congenital thrombocytopathy, thrombasthenia, and various drug-induced platelet defects (9, 10). In each of these conditions, a poor aggregation response to collagen in citrated PRP has been reported; the initial adhesion response has been defined in none. Our demonstration of a normal adhesion response in the aspirin-treated platelet provides increased understanding of this model. Second, the technique provides a basis for better evaluating the effect of platelet age upon platelet function. Hirsh *et al.* (4) presented data suggesting that young plate-

lets react more effectively with collagen. Their conclusions were based upon greater radioactivity appearing on collagen when young platelets were selectively labeled; conversely, when old platelets had the major label, accumulation of label on the collagen was retarded. The present studies indicate that in the presence of adequate collagen, all platelets adhere. Moreover, platelets probably adhere with a single contact and virtually instantaneously. Perhaps the data of Hirsh *et al.* can be explained by the greater size of young platelets (11, 12). Young platelets adhering to collagen, each carrying more label, would give spuriously greater uptake values than an equal number of older platelets.

Summary. A technique is described which measures the adhesion of platelets to connective tissue fragments in platelet-rich plasma (PRP). The principle of the method is measurement of OD in EDTA–PRP to which connective tissue fragments have been added. A fall in OD occurs as the platelets line up on connective tissue fibers. Aspirin-treated platelets adhere normally, although they show reduced aggregation with collagen in citrated PRP. An additional observation is that in the presence of sufficient connective tissue, all platelets participate in the adhesion reaction.

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