Inhibition of Platelet Adhesion to Collagen by Sulfhydryl Inhibitors[1] (35163)

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In the process of hemostasis, platelet adhesion to connective tissue is an essential early event, and collagen is one such material which is highly reactive in this process (1–3). When a blood vessel is injured, platelets adhere to the newly exposed fibers in the connective tissue beneath damaged endothelium. This initial platelet contact, followed by platelet aggregation, eventually leads to the formation of the hemostatic plug associated with activation of the intrinsic blood clotting system (4). Although some of the properties of collagen necessary for platelet interaction have been studied (5, 6), the nature of the binding and the reactive sites on the platelets are unknown.

Earlier studies have shown that sulfhydryl (SH) groups are present on platelets (7) and that they are important in several aspects of normal platelet function (8–10). In this study it is shown that platelet SH groups appear to play an essential role in adhesion to collagen.

Materials and Methods. Blood obtained from healthy donors on no medication was anticoagulated with 0.1 vol of 1% EDTA in isotonic saline. Platelet-rich plasma (PRP) and connective tissue suspension (CTS) were prepared, and specific adhesion was estimated by an optical density method, as previously reported (11).

N-ethyl maleimide (NEM), p-hydroxymercuribenzoate (PHMB), and the poorly penetrating moeity of the latter (10) *p*-hydroxymercuriphenosulfonate (PHMBS) were obtained from Sigma Chemical Co., St. Louis, Mo. L-Cysteine was obtained from Mann Research Labs., N. Y. All items except PHMBS were dissolved in imidazole-buffered saline (IBS), pH 7.3. PHMBS was dissolved in isotonic saline (because it precipitates with imidazole) and the pH was adjusted to 7.3 with 0.1 *N* HCl. PRP was incubated with 0.1 vol each of NEM, PHMB, or PHMBS with or without L-cysteine; the final concentration of the materials tested and the period of incubation is indicated below. Control specimens were incubated with similar volumes of IBS. In each case, 0.1 ml of CTS was added to 1 ml of PRP, and 3 measures of the reaction were taken: Optical density (OD) was recorded; aliquots were taken from the cuvette for examination by phase microscopy; and counts were made of nonadhering platelets. In the last noted measurement, the cuvettes were removed from the instrument after 5 min of stirring, and platelet–CT mixed clumps were allowed to settle for about 5 min. Platelet counts were performed by the method of Brecher and Cronkite (12) on plasma obtained from the extreme top to avoid inclusion of clumps; platelets were similarly counted from the untreated PRP. Generally, there was good correlation between changes in the OD and the platelet count. A significant fall in the OD was associated with more than 60% reduction of the platelet count in the supernatant part of the PRP in the cuvette. This also correlated well with number and size of the platelet–CT clumps seen by phase microscopy.

The effect of sulfhydryl group inhibitors was also studied in a plasma-free system. Platelet buttons were prepared by centrifuging PRP for 25 min in a Clay-Adams Sero-Fuge. The button was washed twice in Gaint-

[1] This study was supported by Grant No. HE 05415-10 from the National Heart Institutes, National Institutes of Health, U. S. Public Health Service.

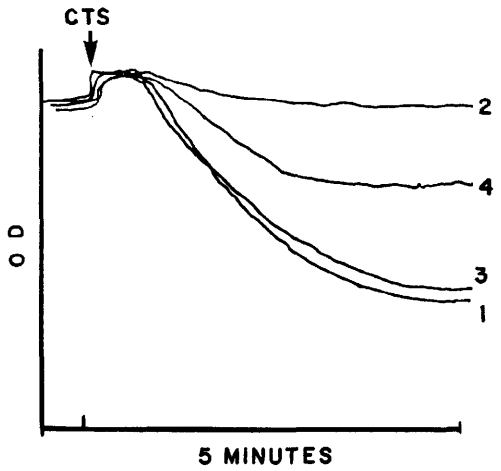

FIG. 1. Platelet adhesion to connective tissue suspension (CTS): (1) control specimen; (2) with NEM; (3) after incubation with L-cysteine and NEM; (4) specimen first incubated with NEM, 5 min later L-cysteine was added.

ner's buffer (13), and was resuspended in buffer to give a platelet count of about 500,000/mm^3. The possible inhibitors were added in the same final concentration as above. At the end of the incubation period a large volume of buffer was added, platelet buttons were prepared again by centrifugation, were washed twice, and were finally resuspended in platelet-poor plasma (PPP). Adhesion to CTS was tested as described below. It was not always possible to resuspend the platelet button in the PPP, and a few clumps were seen in the control specimens.

All experiments were performed with triplicate PRP samples, and with each sample the study was repeated 3 or more times.

Results. NEM, incubated with PRP at a final concentration of 2.5–5 $\times$ 10^{-4} M, strongly inhibited platelet adhesion to CT, as illustrated in Figs. 1 and 2 and Table I. The inhibition was immediate. Lower concentrations of the inhibitor were less effective. PHMB produced the same degree of inhibition at a higher concentration: 1–2 $\times$ 10^{-3} M. There seemed to be a quantitative relationship between the number of platelets in the plasma and the amount of SH inhibitor necessary to produce inhibition, since doubling the platelet number doubled the amount of NEM or PHMB required to inhibit adhesion. Inhibition was not significantly affected by increasing the amount of CTS added. PHMBS, which penetrates platelet membrane slowly (10), showed very little effect immediately at a final concentration of 3 $\times$ 10^{-3} M. With increasing incubation time, however, an inhibitory effect became apparent, and after 45 min, adhesion was markedly reduced (Table I). Platelet aggregation in citrated PRP induced by 2 μM adenosine diphosphate (ADP) or dilute collagen, was strongly inhibited immediately by the same reagent at a final concentration of 10^{-3} M.

The inhibitory effects of NEM or PHMB were completely reversed by L-cysteine added immediately before or simultaneously with either of these agents in equimolar concentration. If added 3 min later it was less effective, and after 7 min it was totally ineffective.

The effect of SH inhibitors on CT was also studied. Aliquots of CTS were incubated for 15 min with NEM or PHMB at the same final concentration. The CT was then washed twice by suspension in a large volume of IBS and centrifugation at 20,000 rpm for 30 min. Such treatment did not affect the reac-

TABLE I. Effect of SH-Inhibitors on Count of Free Platelets.

Specimen	Platelet count $\times$ 10^{3a} Before adding CTS	After adding CTS
1 Control (IBS)	345	72
2 Incubated with NEM 5 $\times$ 10^{-4} M	348	297
3 Incubated with L-cysteine + NEM 5 $\times$ 10^{-4} M	324	65
4 Incubated with PHMB 10^{-3} M	411	369
5 Incubated with L-cysteine + PHMB 10^{-3} M	402	97
6 Incubated with PHMBS 3 $\times$ 10^{-3} M		
a Immediately	312	56
b After 15 min	303	67
c 30 min	311	64
d 45 min	311	199
e 60 min	311	282

[a] Mean of three values.

tivity of CT with platelets. It produced adhesion identical to that of the control specimen incubated with buffer.

NEM or PHMB added to PRP *after* the addition of CTS did not cause any disruption of the adhesion, the fall in the OD was not

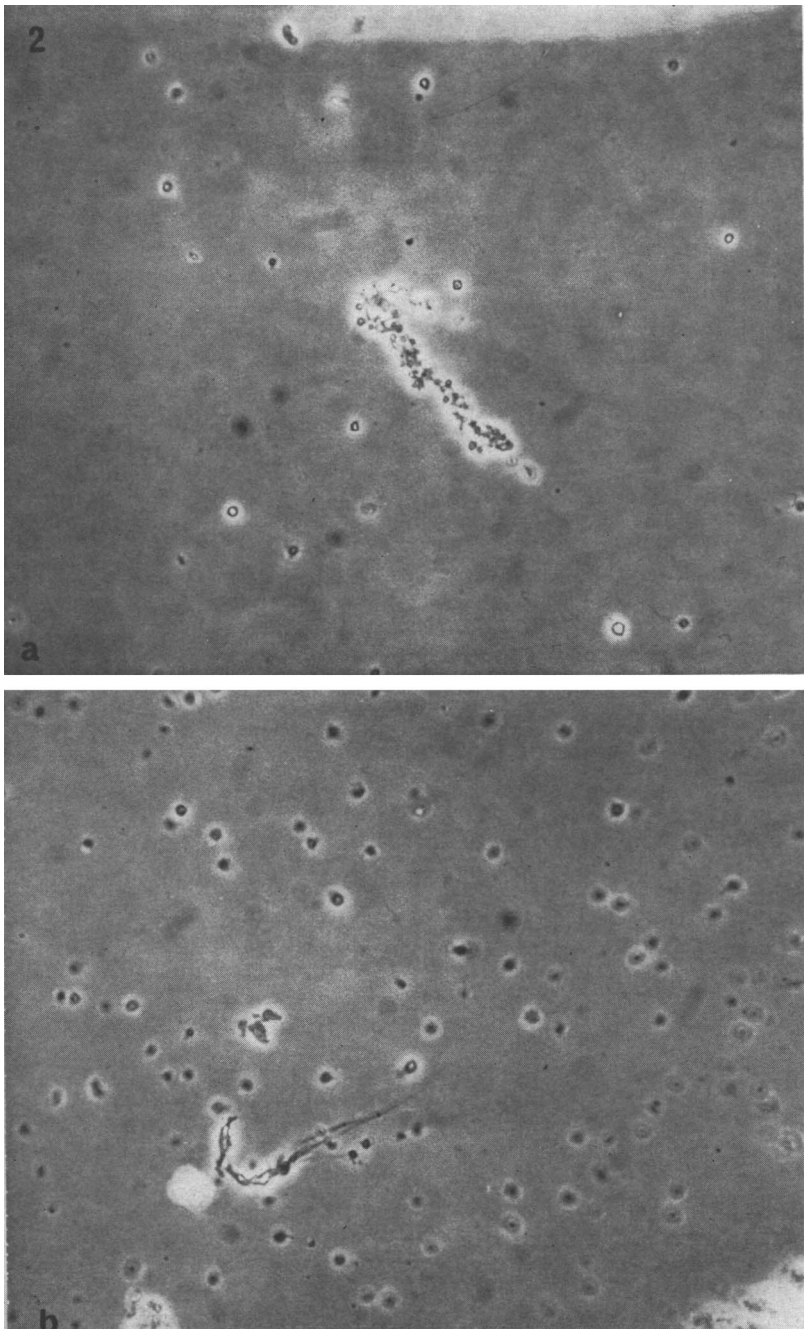

FIG. 2. Phase microphotograph of a specimen of PRP reacted with CTS: (a) control, showing platelets adhering to collagen filaments; (b) PRP incubated with NEM then reacted with CTS. CT fragment is free of platelets; ×520.

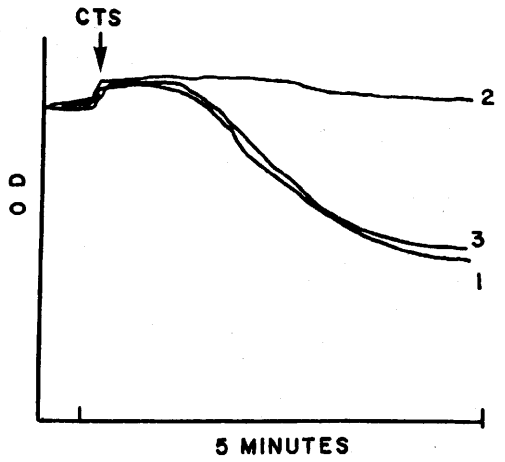

FIG. 3. Effect of NEM on washed platelets: (1) control; (2) with NEM; (3) after incubation with L-cysteine and NEM.

reversed, and the size and number of the platelet–collagen clumps was not affected.

Essentially the same results were obtained with washed platelets exposed to SH inhibitors (Fig. 3).

Discussion. Several investigators have demonstrated that certain aspects of normal platelet function require intact sulfhydryl groups and are altered by SH inhibitors. Robinson *et al.* (8) reported that NEM and *p*-chloromercuribenzoate (PCMB) interfere with the ability of canine platelets to aggregate in respone to ADP or thrombin. Harrison *et al.* (9) showed that human platelet aggregation induced by ADP, ATP, noradrenaline, 5-hydroxytryptamine, or connective tissue extracts was inhibited by NEM and PHMB. More recently, Aledort *et al.* (10) have shown that PCMB and PCMBS inhibit the ADP-induced aggregation of intact platelets and ATPase activity of the lysed cells. Clot retraction was inhibited by PCMB only. In this study we have shown that blocking the sulfhydryl groups of platelets by NEM, a specific SH inhibitor (14), effectively blocks platelet adhesion to collagen. The specificity of the SH group in this reaction was demonstrated by the observation that supplying exogenous thiol groups in the form of L-cysteine completely prevented the effect of the sulfhydryl inhibitors. PHMB, a less specific SH inhibitor (9), produced similar effects at a higher concentration.

Aledort and co-workers (10) have shown that whereas PCMB penetrates intact platelet membranes readily, its sulfonic acid analog, PCMBS, (as with PHMBS) enters platelets slowly, probably because of its strongly hydrophobic sulfonic group. Of interest is the observation that NEM and PHMB exerted an immediate inhibitory effect on adhesion, whereas the inhibition produced by PHMBS was delayed. These findings suggest that platelet–collagen interaction is at least partly dependent on a sulfhydryl-containing protein. However, the failure of PHMBS to exert an immediate effect suggests that the SH inhibition is not simply a matter of interfering with such groups on the platelet surface: Evidently, cell penetration is required for the inhibition. This could mean that internal SH groups are required for normal adhesion; there may also be some indirect effect on platelet membrane resulting from internal SH inhibition. This latter possibility would conform to the observation that the inhibition of platelet adhesion by SH inhibitors was not reversible with subsequent addition of L-cysteine. These findings are in contrast to those obtained for platelet aggregation, where nonpenetrating SH inhibitors were immediately effective. An addition difference was the ability of the inhibitors to disperse aggregated platelets (9), whereas preexisting adhesion was unaffected by these agents.

Summary. The studies reported present evidence that a free or intact sulfhydryl group is involved in platelet adhesion to connective tissue. Exposure of platelets to the readily penetrating sulfhydryl inhibitors NEM or PHMB immediately blocks their ability to adhere to connective tissue. PHMBS, which penetrates platelet membrane very slowly, produces an inhibitory effect only after prolonged incubation. These observations suggest that an SH-containing substrate affecting the adhesion reaction is probably located beneath the plasma membrane of the platelet.

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Received June 18, 1970. P.S.E.B.M., 1970. Vol. 135.