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Potentiation of Platelet Aggregation by Heat-Precipitated Plasma Proteins (40129)

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Human platelet aggregation induced by one agent can be potentiated by another agent. A classical example is the potentiation of adenosine diphosphate-induced aggregation by epinephrine (1). Recently, we found that the addition of small amounts of heat-precipitated plasma proteins to platelet-rich plasma resulted in markedly enhanced platelet aggregation when the mixture was exposed to low concentrations of aggregating agents, but the heat-precipitated plasma protein alone did not cause platelet aggregation.

Materials and methods. Platelet-rich plasma (PRP). Blood was drawn from an antecubital vein of normal volunteers who had not taken any medication in the preceding week. The blood was anticoagulated with buffered sodium citrate (17.64 g trisodium citrate and 7.88 g citric acid in 1 L) in a ratio of nine parts of blood to one part of anticoagulant. PRP was prepared by centrifugation of whole blood at 110*g* for 15 min at room temperature. Washed platelet suspensions (WPS) were prepared from citrated PRP by the method of Rossi (2). The only protein in the wash medium was human albumin (Hyland Co., Costa Mesa, CA; final concentration: 0.5%), which had been heated at 56° for 30 min before use.

Platelet-poor plasma (PPP). PPP was usually prepared by centrifugation of citrated whole blood, or the remaining blood from which PRP had been removed, at 1100*g* for 15 min. Four hemophilic plasma samples (factor VIII coagulant activity <1%) were generously provided by Dr. David Green of Northwestern University. Plasma samples of 5 severe von Willebrand's disease patients (template bleeding time, >20 min; platelet retention in glass bead column, <20% with normal >70%; factor VIII activity, <25%; absence of aggregation of PRP with 1.5 mg/ml ristocetin) were supplied by Dr. Green and Dr. Juan Chediak of Michael Reese Medical Center. One aliquot of the PPP was either placed in an icebath and used immediately, or kept at −20° and thawed once. Two other aliquots of PPP were incubated at 56° for 30 min, and one of the two was centrifuged at 27,000*g* for 15 min to remove heat-precipitated plasma proteins. The supernatant was separated and saved. The precipitate was washed 1–3 times with Tris (0.07 *M*)-saline (0.09 *M*) buffer (pH 7.5) and resuspended in the same buffer to the original volume. The total protein concentration in five plasma samples and in the supernatant of the same plasma samples after heating was determined by the biuret method with "Centrifichem" (Union Carbide, Rye, NY).

Thrombin-clotted plasma (TCP) and serum. TCP was obtained by clotting normal citrated plasma with 1 unit/ml human thrombin (Fibrindex, Ortho Pharmaceutical Co., Raritan, NJ). After the clot was allowed to stand at 37° for 30 min, TCP was separated and placed in plastic tubes. Serum was obtained by clotting whole blood in plain glass tubes for 30 min to 16 hr at 37°. One-tenth volume of sodium citrate (0.1 *M*) was added to some serum samples. Human fibrinogen (Parenogen, Cutter Lab., Berkeley, CA) was dissolved in either saline or serum, 5 mg/ml. Aliquots of TCP, serum or fibrinogen were treated like PPP. The quantities of fibrinogen and/or fibrinogen related antigen materials in four serum and TCP samples were determined by the tanned red cell hemagglutination immunoinhibition (TRCHII) method of Merskey *et al.* (3). Reagents for TRCHII were purchased from the Wellcome Research Lab. (Kent, England).

Platelet aggregation studies. A. *PRP.* An aliquot of 0.3 ml freshly prepared PRP was mixed with 0.05 ml of one of the various fractions described in the preceding two sections. These fractions were serially diluted with appropriate diluents (plasma, serum or Tris buffer) in some experiments. One-half to one minute later, 0.05 ml adenosine diphos-

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phate (ADP, Sigma Co., St. Louis), epinephrine (Upjohn Co., Kalamazoo), or collagen (extracted from human skin (4)) was introduced to the mixture. Weak strengths of aggregating agents causing less than 20% aggregation were used in these experiments. Selected samples were fixed at the end of an aggregation reaction and prepared for electron microscopy (5). In other experiments, several dilutions of heated PPP or heated TCP were added to PRP containing a final concentration of 0.83 m*M* of acetylsalicyclic acid (Sigma) and exposed to concentrations of ADP, epinephrine or collagen that had caused biphasic or substantial aggregation in control samples. A 60- to 90-sec interval was allowed between the addition of ASA to PRP and exposing the mixture to aggregating agents.

B. *WPS.* 0.28 ml of WPS was first mixed with 0.02 ml $CaCl_2$ (final conc. 1 m*M*) and then with 0.05 ml plasma, heated plasma, supernatant or precipitates (washed and resuspended in Tris buffer) of heated plasma, before the mixture was exposed to a weak concentration of collagen.

Fluorescent demonstration of IgG in heat-precipitated plasma proteins. Unwashed precipitates isolated from heated plasma and from heated saline solution of human fibrinogen were fixed in acetone, exposed to rabbit antihuman IgG conjugated with fluorescein isothiocyanate (FITC, Behring Diagnostics, Sommerville, NJ), and examined under a fluorescence microscope. Control samples were first saturated with unconjugated anti IgG before being exposed to FITC-conjugated anti IgG.

Results. Total protein concentration of plasma and of the supernatant of heated plasma; electron microscopic appearance of heat-precipitated plasma proteins. The total protein concentration in five individual plasma samples ranged 4.6–6.1 gm% (av. 5.50 gm%). The protein concentrations in the supernatant of these samples after heating were 4.0–5.8 gm% (av. 4.96 gm%). Transmission electron microscopy revealed similar appearance of heat-precipitated plasma proteins to plasma proteins precipitated by high concentrations of ristocetin (5).

Potentiation of platelet aggregation by heated plasma samples. When fresh human PRP was exposed to low concentrations of ADP, only a small degree of primary aggregation followed by disaggregation was observed. Higher concentrations of ADP induced biphasic or maximum monophasic aggregation. The small degree of aggregation induced by low concentrations of ADP was augmented by the addition of less than 0.4% (v/v) of heated (56°, 30 min) autologous or homologous normal plasma (Fig. 1). The marked platelet aggregation of samples containing heated plasma was confirmed by the demonstration of large platelet aggregates by electron microscopy. The heated plasma itself, up to 12.5% (v/v) of the reaction mixture, did not cause platelet aggregation. In addition to heated normal plasma, a number of other heated plasma samples including thrombin-clotted plasma (TCP), hemophilic plasma and von Willebrand plasma also showed similar potentiation. However, neither heated serum (uncitrated or citrated) nor a heated commercial human fibrinogen preparation had the potentiation effect. When fibrinogen was dissolved in serum and then heated, the heated preparation showed marked potentiation effect. The ability of heated plasma samples to enhance platelet aggregation was also observed when epinephrine or collagen was the aggregating agent. In addition, heated plasma also potentiated collagen-induced aggregation of washed platelet suspensions (WPS) (Fig. 2). It should be noted that visible precipitates were present in all heated samples except heated serum. Fibrinogen and/or fibrinogen-related antigen was 2.5 μg/ml in three serum samples, and 3.75 μg/ml in one; in all 4 TCP samples, the levels were >320 μg/ml. If the heat-precipitated plasma proteins were removed, the supernatant solution lost the ability to potentiate platelet aggregation (Fig. 3). However, precipitates resuspended in buffer retained the activity (Fig. 4). Precipitates that had been washed three times potentiated aggregation as well as those washed only once.

Counteraction of effect of ASA by heated plasma. The presence of 0.83 m*M* ASA in PRP inhibited the secondary aggregation induced by ADP and epinephrine, and the aggregation by collagen. The aggregation of ASA-treated PRP could be augmented by increasing the concentration of ADP and, to

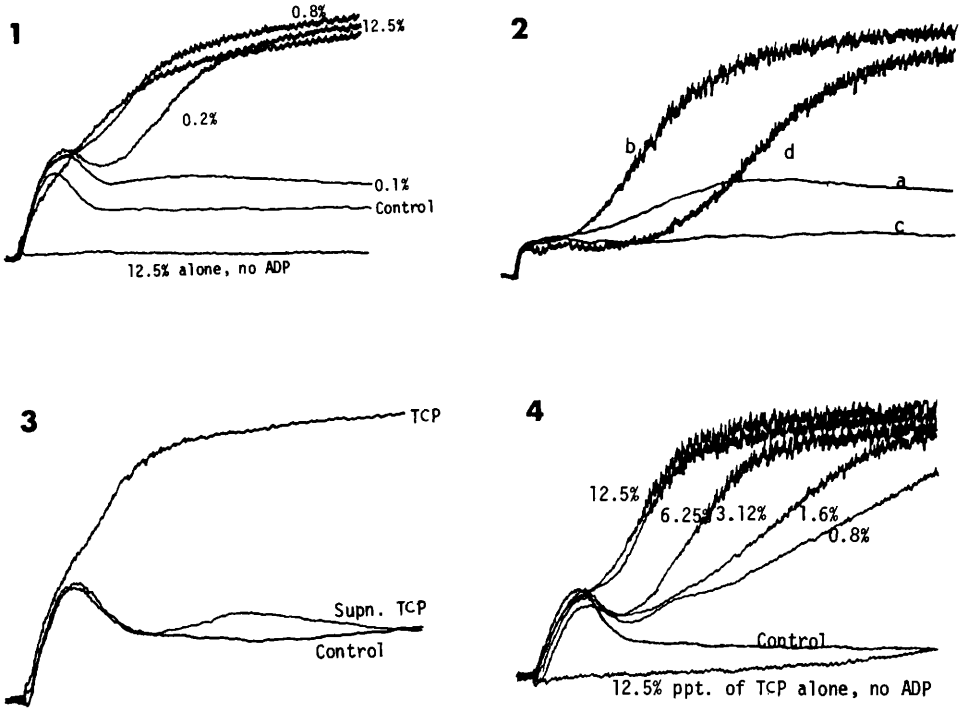

FIG. 1. Light transmission tracings depicting potentiation of ADP-induced aggregation of human PRP by various concentrations of heated normal plasma. Numerals in this and subsequent figures indicate final concentrations (% v/v) of heated plasma in reaction mixture.

FIG. 2. Potentiation of collagen-induced aggregation of WPS by heated plasma (Curve b, 12.5%) and precipitates of heated plasma (Curve d). Unheated plasma (Curve a) and the supernatant of heated plasma (Curve c) do not show such an effect.

FIG. 3. Platelet aggregation curves showing potentiation of ADP-induced aggregation by 12.5% heated TCP (thrombin-clotted plasma). The supernatant of heated TCP is ineffective.

FIG. 4. Potentiation of ADP-induced aggregation of PRP by various concentrations of precipitates of heated TCP. The precipitates were washed once and resuspended in Tris buffer. Dilutions of TCP were made with the same buffer.

a much lesser extent, the concentration of collagen. A 16-fold increase in epinephrine concentration, from 1.5 to 25 μM, often failed to increase even the primary aggregation of ASA-treated PRP. However, ASA-treated PRP exhibited biphasic aggregation with ADP or epinephrine, and marked aggregation with collagen in the presence of 3 to 6% of heated plasma (Fig. 5).

Comparable fluorescent staining in heated plasma and fibrinogen samples. Nonspecific fluorescent staining was observed in heat-precipitated plasma proteins treated with anti-human IgG conjugated with fluorescein isothiocyanate (FITC), as well as in heated plasma first exposed to unconjugated anti IgG before treated with FITC-conjugated anti IgG. Similar nonspecific staining was found in heat-precipitated fibrinogen originally dissolved in saline.

Discussion. We have shown that the inclusion of small amounts of heated normal plasma in fresh human PRP or WPS, or PRP previously treated with ASA, resulted in a marked potentiation of platelet aggregation when the mixture was subsequently exposed to low concentrations of ADP, epinephrine or collagen. Plasmas obtained from hemophiliacs and von Willebrand patients, and those clotted by thrombin were equally effective after heating. Although heated serum (citrated and uncitrated) or a commercial fibrinogen dissolved in saline was ineffective, the heated serum–fibrinogen mixture was.

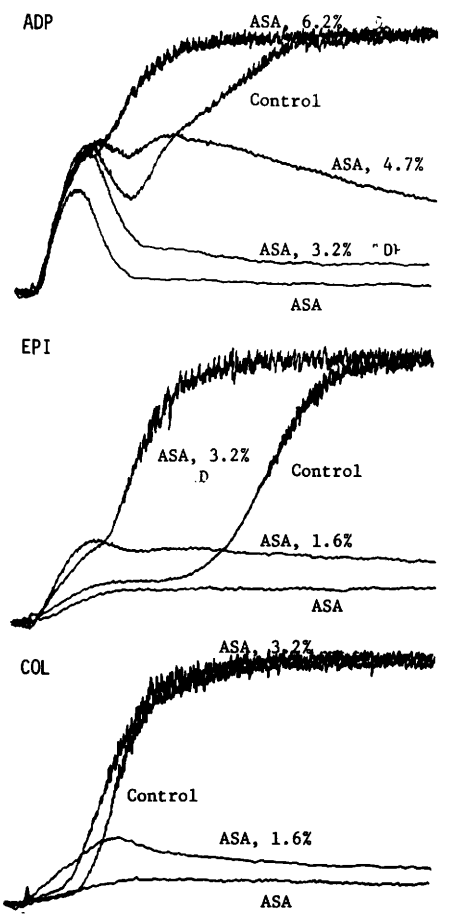

FIG. 5. Obliteration of the inhibitory effect of ASA on platelet aggregation by heated TCP present in the reaction mixture. Final concentration of ASA in all samples, except the controls, is 0.83 m*M*. % indicates final concentrations of TCP in reaction mixture.

The active platelet aggregation potentiating elements reside with the precipitates of heated plasma. These elements should be largely composed of heat-clumped fibrinogen. Since fibrinogen dissolved in saline did form precipitates after heating but did not potentiate platelet aggregation, the effect of heated plasma cannot be attributed to fibrinogen alone, or to the presence of particulate matter in the reaction mixture. Many of our findings contain certain parallelisms to the observation of Zucker and Grant (6), who found that the ability of zymosan to induce human platelet release is conferred by incubating zymosan with plasma, TCP or a serum–fibrinogen mixture, but not with serum alone. In our study, the relationship between heat-precipitated fibrinogen and serum is not clear. One possible explanation is that the heat-precipitated fibrinogen serves as a carrier or an adsorbing surface for a platelet aggregation potentiating factor which is present in serum. The serum factor may be inactive until it has reacted with or is adsorbed on fibrinogen clumps. The reason that heated TCP was effective while heated serum was not appears to be that fibrinogen was not completely clotted by thrombin in the former whereas it was completely consumed in the latter. Indeed, Zucker and Grant (6) found fibrinogen and fibrinogen-related antigen materials in TCP but not in serum. Their findings are substantiated by our results. Apparently, the precipitates present in the heated TCP are derived from the unclotted fibrinogen.

While the identity of the active elements remains unknown, it is not any of the clotting factors that is consumed in clotting and/or inactivated by heating. It is not the von Willebrand factor either. One serum factor we did strongly suspect was IgG. IgG aggregates are known to potentiate ADP-induced aggregation of WPS, but apparently not of PRP (7–9). Although most of our experiments were performed in PRP, we felt the question of IgG deserved some investigation. Heating is likely to cause IgG to form aggregates; these aggregates might have coprecipitated with fibrinogen and been responsible for the observed phenomenon. The failure to demonstrate specific staining of IgG in heat-precipitated plasma proteins using a highly sensitive fluorescent technique seems to have precluded that possibility, however.

The effect exerted by heat-precipitated plasma proteins does not appear to involve complement action. Potentiation of aggregation was observed in the WPS system in which all protein materials had been decomplemented by heating. The possibility that complement components may be present on the surface of washed platelets cannot be ruled out. Whether such components are indeed present on washed platelets is still a matter of controversy (8, 10, 11). There is little doubt that the net result of the effect of heat-precipitated plasma proteins is to affect the platelet release mechanism. Apparently,

when the release mechanism is inhibited by ASA, it can still be affected by heat-precipitated plasma proteins. As to where precisely the heat-precipitated proteins exert their effects on a rather complicated platelet release pathway remains to be investigated.

Summary. Aggregation of human PRP by low concentrations of ADP, epinephrine or collagen, and of human WPS by collagen, was markedly potentiated if the PRP or WPS was premixed with small amounts of heated (56°, 30 min) normal plasma, thrombin-clotted plasma, hemophilic or von Willebrand plasma. Heated plasma samples by themselves did not induce platelet aggregation. The platelet aggregation potentiating activity of these samples resides with the heat-precipitated proteins. Neither serum nor a saline solution of fibrinogen was effective. However, the serum solution of fibrinogen was effective after heating. The inhibitory effect of aspirin was completely counteracted by small quantities of heated plasma in the reaction mixture. The effect of heat-precipitated plasma proteins appears to be on the platelet release mechanism, making it highly sensitive to minute amounts of aggregants.

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