Effect of Sulfhydryl Inhibitors on Platelet Agglutinability.* (28512)

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Thrombin and adenosine diphosphate (ADP) induce platelet agglutination in the presence of appropriate divalent cations(1, 2). The means by which these substances induce agglutination, and the relationship of agglutination to alterations in enzymatic activities of platelets are controversial. Through the use of sulfhydryl reagents and inhibitors, intact thiol groups have been demonstrated to be essential for maintenance of platelet integrity(3,4) and for certain enzymatic and biological functions of platelets(5,6,7). However, the role of thiol groups in platelet agglutination is not clear. Grette found no inhibition of platelet viscous metamorphosis with the mercaptide forming agent Mersalyl at 35°C. With lower temperatures, a delay in the appearance of platelet aggregation was observed with Mersalyl(7). Zucker and Borrelli reported that mercuric chloride inhibited the thrombin-produced viscous metamorphosis of washed platelets(8). In platelet-rich plasma, platelet aggregation induced by thrombin or ADP was inhibited by 2.5×10^{-3} M mercuric chloride, but not by 2.5×10^{-5} M mercuric chloride or the thiol alkylating agent iodoacetate(9). The present study describes alterations in the ability of platelets to agglutinate with thrombin and ADP, in the presence of calcium, when platelets are incubated with certain sulfhydryl inhibitors.

Materials and methods. Adenosine diphosphate, N-ethyl maleimide (NEM), and the sodium salt of p-chloromercuribenzoic acid (PCMB) were acquired from Mann Research Laboratories. Methyl mercuric nitrate (MMN) was prepared from recrystallized methyl mercuric iodide (Columbia Organic Chemicals Co.) by equimolar reaction with silver nitrate in methanol. The silver iodide which formed was removed by filtration and the product was crystallized twice from methanol. Tris buffer, 0.05 M tris-hydroxymethyl aminomethane, pH 7.4, was made isotonic with sodium chloride. It was used to prepare all inhibitor solutions and for making 22 mM solutions of $CaCl_2$ and $MgCl_2$ from 0.22 M aqueous cation solutions. The solvent for ADP and thrombin was 0.154 M NaCl. Topical thrombin (Parke Davis & Co.) was partially purified on a G-25 Sephadex column equilibrated with normal saline. Thrombin unitage is expressed in Iowa units (10). Standard suspensions of washed canine platelets were prepared at 4°C from canine plasma collected in isotonic, pH 7.4, ethylenediamine tetra-acetic acid(11). The washed platelets were suspended at a concentration of 400,000/cmm in a solution of 0.0054 M sodium citrate and 0.146 M NaCl at 4°C. Platelet suspensions were kept in an ice bath and used within 4 hours of preparation. Only platelet preparations which gave 4+ agglutination with both thrombin (1.0 U/ml, final concentration) and ADP were used. All glassware was silicone-coated (G. E. Dri-Film SC-87) except the test tubes in which agglutination tests were performed.

The macroscopic platelet agglutination test of Brinkhous *et al.*(11) was modified and used as follows. The test was performed in 2 stages. In the first or incubation stage, 0.2 ml of platelet suspension was incubated at 28°C with 0.05 ml of varying concentrations of an inhibitor for specified time periods. In the second or agglutination stage, 0.05 ml of 22 mM calcium chloride and 0.1 ml of thrombin or ADP were added to the platelet-inhibitor mixture. The addition of thrombin or ADP terminated the incubation stage and initiated the agglutination stage. The time at which agglutination was first seen was recorded. Degree of platelet agglutination was graded macroscopically, 2 minutes after initiation of the agglutination stage. This grading was immediately confirmed with phase microscopy(11).

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Results. Cation requirement for ADP-induced platelet agglutination. In control experiments it was found that ADP alone failed to induce agglutination of washed canine platelets. This is the same result that Käser-Glanzmann and Lüscher reported with

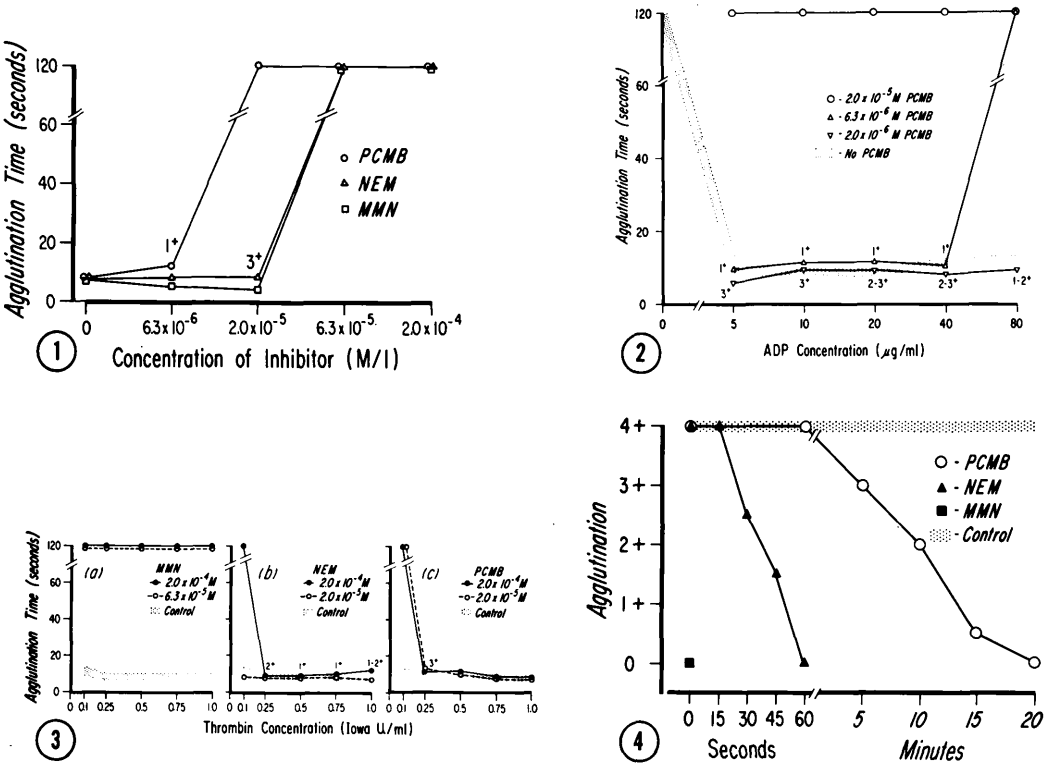

FIG. 1. Comparison of inhibitory effects of PCMB, MMN, and NEM on platelet agglutination induced by Ca^{++} (2.7 mM/l) and ADP at a final concentration of 10 μg/ml (2.3×10^{-6} M). Two parts of platelet suspension were incubated with 0.5 part of inhibitor for 30 sec., then 0.5 part of calcium and 1.0 part of ADP were added to initiate agglutination. Time at which agglutination was first seen is plotted. Unless otherwise indicated, points at 120 sec are zero agglutination and all other points are 4+ agglutination. Inhibitor concentrations expressed above are those in platelet-inhibitor incubation mixture. All control tests with tris saline substituted for Ca^{++} gave no agglutination. Controls containing only platelets and inhibitors were negative. When saline was substituted for ADP, agglutination occurred only in controls with 6.3×10^{-6} M and 2.0×10^{-6} M MMN.

FIG. 2. Inhibitory effect of PCMB on platelet agglutination induced by Ca^{++} and varying concentrations of ADP. Conditions for tests identical to those in Fig. 1. ADP concentrations are those in final agglutination mixture. Agglutination was absent in controls lacking either Ca^{++} or ADP.

FIG. 3. Comparison of inhibitory effects of PCMB, MMN and NEM on platelet agglutination induced by Ca^{++} (2.7 mM/l) and varying concentrations of thrombin. Two parts of platelet suspension were incubated with 0.5 part of inhibitor (final concentration of inhibitor is that in incubation mixture) for 30 sec. Following this 30 sec incubation period, 0.5 part of calcium and 1.0 part of thrombin were added to initiate the agglutination stage. Thrombin concentration is that in final agglutination mixture. Time at which agglutination was first seen is plotted. Unless otherwise indicated, points at 120 sec are zero agglutination and all other points are 4+ agglutination. For controls plotted above, platelets were incubated with tris buffer in place of an inhibitor. Platelet agglutination was absent in controls lacking either Ca^{++} or thrombin.

FIG. 4. Effect of varying incubation time of platelets with 2.0×10^{-4} M conc of each inhibitor on agglutination induced by 1.0 U/ml thrombin and Ca^{++}. Platelets were incubated with 2.0×10^{-4} M conc of each inhibitor at 28°C prior to induction of agglutination with Ca^{++} (2.7 mM/l) and 1.0 U/ml thrombin. Agglutination is plotted as degree of agglutination 2 min after initiation of agglutination stage. In control test, tris saline was substituted for inhibitor in incubation stage.

washed human platelets(12). However, in the presence of 2.7 mM Ca^{++} or Mg^{++}, ADP induced prompt platelet agglutination (7-10 sec) which was graded 4+. Controls with Ca^{++} but without ADP were negative. Rapid agglutination was never seen in the Mg^{++} controls without ADP. However, the Mg^{++} controls were often positive by the end of 2 minutes. The cation requirement for ADP induced agglutination of washed platelets extends the findings of Born with platelet-rich plasma(2).

Effect of PCMB, NEM, and MMN on platelet agglutination induced by ADP and Ca^{++}. Varying concentrations of the 3 sulfhydryl inhibitors were incubated with platelets for 30 seconds before addition of Ca^{++} and ADP. The results shown in Fig. 1 demonstrate that PCMB at 6.3×10^{-6} M partially inhibited platelet agglutination. At greater concentrations of PCMB inhibition appeared complete. With NEM, inhibition was complete at 6.3×10^{-5} M or greater. The results with MMN were more complex. Complete inhibition was seen with MMN concentrations of 6.3×10^{-5} M or greater, but with lower concentrations there was both an acceleratory and intensifying effect on ADP-induced platelet agglutination. In controls lacking ADP, 6.3×10^{-6} M and 2.0×10^{-5} M MMN induced rapid and intense agglutination of platelets in the presence of Ca^{++}. MMN alone did not induce agglutination.

Effect of different concentrations of PCMB on platelet agglutination induced by Ca^{++} and varying amounts of ADP. Inhibition of agglutination was seen with PCMB at a lower molar concentration than with the other inhibitors. Therefore, further studies were conducted to determine whether inhibition by PCMB at a given concentration could be overcome with an increase in ADP concentration, Fig. 2. It is evident that the inhibitory effect of PCMB on platelet agglutination was not overcome by increasing ADP concentration over a 16-fold range. This held true even for the weakest inhibitory concentration of PCMB. It is evident also that increasing PCMB concentration lowers the degree of agglutination with ADP without affecting the agglutination time.

Effect of PCMB, NEM and MMN on platelet agglutination induced by Ca^{++} and thrombin. The results obtained by varying the thrombin concentration and keeping the inhibitor concentration constant are given in Fig. 3. Each inhibitor was used at 2 concentrations. With a 30 second incubation period, increasing thrombin concentration had no apparent effect on inhibition produced by MMN (Fig. 3a), and little effect on the inhibition caused by NEM (Fig. 3b). Both concentrations of PCMB inhibited platelet agglutination induced by weak thrombin (0.1 U/ml), but failed to inhibit platelet agglutination induced by stronger thrombin, Fig. 3c. As a control, 5.0 U/ml thrombin and 1.0 U/ml thrombin were incubated in siliconed tubes for 1 hour at 28°C with 9.0×10^{-4} M PCMB and MMN, and 9.0×10^{-3} M NEM. No loss in thrombin activity was demonstrated.

The effects of varying incubation periods on platelet agglutination induced by 1.0 U/ml thrombin are shown in Fig. 4. To permit comparison, all inhibitors were used at 2×10^{-4} M. Between 15 and 20 minutes incubation was required for PCMB to inhibit completely platelet agglutination induced by 1.0 U/ml thrombin and Ca^{++}. About 1 minute incubation was required for similar action by NEM. With shorter incubation of platelets with NEM, the platelets were observed to undergo transient agglutination followed by deagglutination. Without incubation, 2.0×10^{-4} M MMN inhibited thrombin-induced platelet agglutination, as measured microscopically at 2 minutes. In some of these experiments with MMN, transient agglutination occurred 5 to 10 seconds after addition of thrombin and Ca^{++}, but deagglutination occurred by 60 seconds. In a few experiments, incubation periods of up to 45 seconds were required to eliminate completely this transient agglutination. Platelet clumps resulting from agglutination induced by 1.0 U/ml thrombin and Ca^{++} were observed to undergo deagglutination if MMN (final concentration 1.25×10^{-4} M) was added within

30 seconds of the time agglutination was first seen.

Discussion. The results of this study demonstrate clearly the inhibitory effects of sulfhydryl inhibitors on platelet agglutination. Two other interesting effects are described: the action of weak MMN in causing platelet agglutination in presence of Ca^{++}, and the deagglutination of platelet clumps produced by MMN and NEM under certain circumstances.

Incubation of platelets with the inhibitors is required for inhibition of thrombin-induced agglutination (Fig. 4). This strongly suggests that the action of the inhibitors is on the platelets. An increase in platelet breakdown on incubation with sulfhydryl inhibitors has been shown previously(4). With the short incubation periods of platelets with inhibitors used in our experiments, little change in platelet number was observed. Of the sulfhydryl inhibitors studied by Koppel *et al.*, NEM was the least effective in producing platelet breakdown(4). However, NEM was very active in inhibiting platelet agglutinability (Fig. 4). It appears that the inhibition of platelet agglutination by sulfhydryl inhibitors cannot be explained on the basis of platelet breakdown.

PCMB, MMN and NEM rapidly inhibit the ability of platelets to agglutinate with Ca^{++}, and ADP or 0.1 U/ml thrombin. When agglutination is induced by 1.0 U/ml thrombin, PCMB inhibition appears slowly with incubation; MMN and NEM inhibition is complete within seconds, Fig. 4. The difference in inhibition of agglutination induced by 1.0 U/ml thrombin may be explained, in part, by different rates of diffusion of the three inhibitors into the platelets. It has been demonstrated that PCMB only slowly diffuses across red cell membranes whereas NEM diffuses readily(13).

Inhibition of platelet agglutinability by the sulfhydryl inhibitors does not distinguish *per se* between the enzymatic or non-enzymatic nature of platelet thiol groups which apparently are involved directly in agglutination. Organic and inorganic mercurials at low concentrations increase the activity of some enzymes under certain conditions(14,15). The enhancing effect of weak MMN on platelet agglutinability may be similar to the observed increase in myosin adenosine triphosphatase activity with partial inhibition of thiol groups of myosin(14). This enhancing effect and the deagglutinating effect of MMN and NEM suggest that the inhibitors are reacting with thiol groups of an enzyme(s) involved in platelet agglutination.

Summary. 1. In the presence of Ca^{++} or Mg^{++}, ADP induces agglutination of platelets washed at 4°C. 2. The sulfhydryl inhibitors PCMB, NEM, and MMN inhibit the ability of washed canine platelets to agglutinate with thrombin or ADP in the presence of Ca^{++}. 3. The effectiveness of PCMB in inhibiting platelet agglutinability is dependent upon thrombin concentration used to induce agglutination. The inhibitory action of PCMB is independent of changes in ADP concentration. 4. MMN causes deagglutination of platelets from clumps formed in the presence of 1.0 U/ml thrombin and Ca^{++}, if the MMN is added to the platelet clumps within 30 seconds of agglutination. 5. MMN, at non-inhibitory concentrations (2.0×10^{-5} M or less), induces rapid and intense platelet agglutination in the presence of Ca^{++}.

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A Simplified Method for Bactericidal Assay of Natural Antibodies Against Gram-negative Bacteria. (28513)

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It is now established that the bactericidal activity of fresh normal serum against Gram-negative bacteria is attributable to a system comprised of specific antibody, complement, and divalent cation(1,2). We have applied this knowledge to the measurement of natural antibodies in normal serum by using a complement source freed of antibody and appropriate sensitive strains of Gram-negative bacteria(3). The test serum restores bactericidal activity to the system to the extent that it contributes specific antibody. This assay has provided useful information as to the origin, specificity, and distribution of natural antibodies to Gram-negative bacteria (4) and the effects of endotoxin on the levels of natural antibodies in mice(5).

However, there are factors which tend to limit the general usefulness of this method (3), *viz.* (a) use of human serum as a complement source, which necessitates removal of natural antibody by absorption with suspensions of Gram-negative bacteria and (b) use of an inoculum of 10^6 bacteria which requires the plating out of an extended series of decimal dilutions of bacteria and serum to enumerate the survivors. This is time consuming and requires large numbers of pipettes and test tubes.

Modifications of the method have been devised to make the test more practical for general use and to increase its overall efficiency. Precolostral calf serum was employed as a complement source lacking in certain natural antibodies(6), and the bacterial inoculum was reduced to 10^2 cells. These changes, together with modifications in the performance of the assay, provided a rapid and simple method without any loss in precision.

Materials. Calf serum. Newborn calves of Holstein and Guernsey breeds were removed from their mothers prior to suckling and exsanguinated.* The blood was allowed to stand at room temperature for 1-3 hours to facilitate clotting, then kept at 4°C overnight. The serum was separated by centrifugation at 4°C and stored in rubber stoppered serum bottles at −50°C. Individual samples were thawed as needed; unused portions were discarded.

Rabbit serum. Adult New Zealand white rabbits were bled by cardiac puncture, and the serum from 18 rabbits was pooled and stored at −30°C.

Bacterial cultures. The various test strains of *Enterobacteriaceae,* susceptible to the bactericidal action of fresh human serum(3) were from the culture collection of the Walter Reed Army Institute of Research made available through the courtesy of Mr. A. Abrams: *Salmonella typhosa* 0901; *Shigella dysenteriae* (Type 1); *Escherichia coli* 0127 B:8; *Pseudomonas aerugenosa* (25-A-2); *Aerobacter aerogenes* (Type 1 NCTC #418); and *Klebsiella pneumoniae* K-12 (35-A-38). All were smooth cultures, not agglutinated by acriflavin or by the boiling of

* These sera were obtained for us from dairy herds in Maryland and Ohio by Mr. Robert Benson, Division Research Services, Nat. Inst. Health, and Dr. Robert Henthorne, Laboratory Animal Facilities, Ohio State Univ., Columbus, respectively. Sera obtained from herds in Colorado were purchased from Colorado Serum Co., Denver.