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Platelet C1- inhibitor. A secreted alpha-granule protein.

A H Schmaier, … , P M Smith, R W Colman

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Research Article

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Platelet C1 Inhibitor

A Secreted Alpha-Granule Protein

Alvin H. Schmaier, Paulette M. Smith, and Robert W. Colman
Hematology/Oncology Section, Department of Medicine, Thrombosis Research Center, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Abstract

In order to characterize which proteins of the contact phase of coagulation interact with platelets, human platelets were studied immunochemically and functionally to determine if they contain C1 inhibitor. By means of monospecific antibody to C1 inhibitor, a competitive enzyme-linked immunosorbent assay (CELISA) was developed to measure directly platelet C1 inhibitor. With the CELISA, from 33 to 115 ng of C1 inhibitor antigen per 10^8 platelets from 15 normal donors was quantified in lysates of washed human platelets solubilized in nonionic detergent. The mean concentration in 10^8 platelets was 62±33 ng (SD). Plasma C1 inhibitor either in the platelet suspension medium or on the surface of the platelets could account for only from 6.5 to 16% of the total antigen measured in the solubilized platelets. Upon functional studies, platelets contained 84±36 ng (SD) of C1 inhibitor activity in 10^8 platelets. As assessed by the CELISA, platelet C1 inhibitor antigen was immunochemically identical to plasma and purified C1 inhibitor. In contrast, the mean concentration of platelet C1 inhibitor antigen in platelets from four patients with classical hereditary angioedema was 8.3 ng/10^8 platelets (range, 5.3 to 11.3 ng/10^8 platelets). 25 and 31% of the total platelet C1 inhibitor was secreted without cell lysis from normal platelets after exposure to collagen (20 μg/ml) and thrombin (1 U/ml), respectively, and this secretion was blocked by metabolic inhibitors. Platelet subcellular fractionation showed that platelet C1 inhibitor resided mostly in alpha-granules, similar to the location of platelet fibrinogen. Thus, human platelets contained C1 inhibitor, which became available by platelet secretion. The identification of platelet C1 inhibitor suggests that platelets may modulate the activation of the proteins of early blood coagulation and the classical complement pathways.

Introduction

Understanding of the role of human platelets in the activation of plasma coagulation proteolysis has been furthered by the recognition that four plasma hemostatic cofactors—fibrinogen (1–3), Factor V (4–8), Factor VIII-related antigen (9–11), and high molecular weight kininogen (12)—are contained within platelets. Similarly, the details of inhibition of coagulation and fibrinolysis reactions on or close to the platelet surface can begin to be formulated since three of the major plasma protease inhibitors, alpha-2-macroglobulin (13), alpha-1-antitrypsin (13, 14), and alpha-2-antiplasmin (15–20), are also platelet constituents. A recent preliminary report indicated that C1 inhibitor (C1 INH)[1] antigen detected by immunofluorescence is present on or in platelets (21). Since C1 INH is a major plasma proteolytic inhibitor of the proteases of the initial phase of intrinsic coagulation (kallikrein, activated Factor XII, and Hageman factor fragment) (22–25) and the classical complement system (C1r and C1s) (26), we initiated a study to determine whether C1 INH is contained with platelets. This study presents immunochemical and functional evidence for the presence of C1 INH in platelet granules and its secretion after platelet activation with collagen and thrombin.

Methods

Materials. Antisera to fibrinogen were purchased from Atlantic Antibodies (Scarborough, ME). Nagarse was purchased from the Enzyme Development Corp. (New York). Acid soluble collagen (1 mg/ml) was purchased from Hörmon-Chemie (Munich). Human alpha-thrombin (3125 U/mg) was a kind gift of Dr. J. Fenton, New York State Department of Health (Albany, NY). Na ^{125}I (50 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Iodogen (chloroamide,1,3,4,6-tetrachloro-3alpha-6alpha-diphenylglycoluril) was obtained from Pierce Chemical Co. (Rockford, IL). Sodium barbital buffer, pH 8.8 was purchased from Gelman Sciences, Inc. (Ann Arbor, MI). Agarose of the highest electroendosmotic grade was obtained from FMC Corp., Marine Colloids Div. (Springfield, NJ). Firefly luciferase was purchased from Dupont, Inc. (Wilmington, DE). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Plasma and platelets. C1 INH-deficient plasma from a patient with classical hereditary angioedema (C1 INH activity and antigen level, 30 μg/ml [27]) and platelets from four other patients with classical hereditary angioedema with plasma C1 INH activity and antigen levels of 16 and 20 μg/ml, 52 and 54 μg/ml, 32 and 69 μg/ml, and 50 and 63 μg/ml, respectively, were directly donated to us. Pooled normal plasma (lots 120 and N10) was purchased from George King Biomedicals, Inc. (Overland Park, KS). Normal donors were males and females (aged 21–40) on no medications, including oral contraceptives, who gave their written informed consent. Fresh blood was collected, and platelet-rich plasma and platelet-poor plasma were prepared as previously described (28).

Preparation of C1 INH. C1 INH was purified from plasma by polyethylene glycol precipitation, DEAE-cellulose chromatography, and Concanavalin A-Sepharose affinity chromatography according to the method of Sim and Reboul (29). Fractions containing C1 INH were detected antigenically by counterimmunoelectrophoresis (30). This preparation did not for us result in a homogenous protein upon sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Further

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1. *Abbreviations used in this paper:* C1 INH, C1 inhibitor; CELISA, competitive enzyme-linked immunosorbent assay; EID, electroimmunodiffusion; RID, radial immunodiffusion.

purification was done by performance of gel filtration over Sepharose CL-6B. Fractions containing $C\bar{1}$ INH antigen by counterimmunoelectrophoresis (30) constituted a single peak of ~220,000 M_r by gel filtration. By nonreduced SDS gel electrophoresis (31), purified $C\bar{1}$ INH was a single component (M_r 107,000) (Fig. 1). Upon reduction with 2% beta-mercaptoethanol there was a slight decrease in size (M_r 100,000) of the major band and the appearance of two minor components (M_r 62,000 and 46,000), which constituted <5% of the Coomassie Blue stained material by densitometer scanning. These minor components probably represented degradation products of $C\bar{1}$ INH as previously reported (29). As shown by immunodiffusion (32) the purified protein was not contaminated with human serum albumin and transferrin.

Protein determinations were performed by the methods of Bradford (33) using crystalline bovine serum albumin as the standard, and of Scopes (34).

Purified $C\bar{1}$ INH was radiolabeled with Na ^{125}I by the use of Iodogen by the method of Fraker and Speck (35). Purified $C\bar{1}$ INH (50–200 μg in 0.02 M Tris, 0.15 M NaCl, pH 8.0) was incubated with carrier-free Na ^{125}I in a plastic vial precoated with Iodogen (1–4 μg) for 15–35 min. The iodination reaction was stopped by the addition of sodium metabisulfite (50 μg/ml final concentration), and free ^{125}I was separated from protein-bound ^{125}I by gel filtration on a 0.8 × 10 cm column of Sephadex G-50 equilibrated in 0.02 M Tris, 1.0 M NaCl, pH 8.0 containing 0.25% gelatin. The specific radioactivity of the protein varied from 3 to 10 μCi/μg. The radiolabeled protein retained its full antigenic properties.

Antisera and antibodies. Antisera and antibodies to $C\bar{1}$ INH (lots CE1-011-1 and CE1-IGG-006-3 from Atlantic Antibodies) were monospecific to purified $C\bar{1}$ INH and $C\bar{1}$ INH in plasma upon double immunodiffusion (32), forming a single precipitin arc which showed complete identity. These antisera and antibodies did not cross-react upon immunodiffusion with any preparation of human and bovine serum albumin used in these studies.

Hereditary angioedema plasma was rendered totally deficient in $C\bar{1}$ INH antigen by anti-$C\bar{1}$ INH affinity chromatography and is hereafter referred to as totally $C\bar{1}$ INH-immunodeficient plasma. Antibody to $C\bar{1}$ INH was covalently linked to cyanogen bromide-activated-Sepharose 4B. 7 ml of fresh-frozen hereditary angioedema plasma were applied to a 1-ml anti-$C\bar{1}$ INH-Sepharose 4B column and fractions, that did not adhere to the column, and were the least diluted with buffer, were collected in 0.5-ml aliquots and rapidly refrozen at −70°C. This artifically produced, totally $C\bar{1}$ INH-immunodeficient plasma did not form a precipitin arc with anti-$C\bar{1}$ INH antibody upon immunodiffusion, and upon a competitive enzyme-linked immunosorbent assay (CELISA) for $C\bar{1}$ INH antigen produced no competitive inhibition, indicating that the $C\bar{1}$ INH concentration in the artificially depleted plasma was <25 ng/ml (see below).

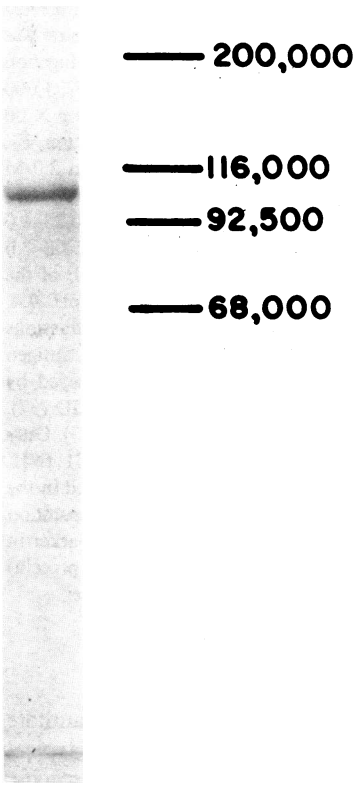

Figure 1. Purified $C\bar{1}$ INH (10 μg) was electrophoresed on a 7.5% nonreduced SDS-polyacrylamide gel.

Assays. Antigenic $C\bar{1}$ INH was quantified by radial immunodiffusion (RID) (36) and electroimmunodiffusion (EID) (37). RID was performed on 74 × 50 mm glass plates covered with 7 ml of 1% agarose in sodium barbital buffer pH 8.8, $\tau/2 = 0.0375$ containing 1% antiserum. Samples were placed into 2.5-mm diam wells and allowed to diffuse for 60 h. EID was performed as previously reported (12). In all instances where purified $C\bar{1}$ INH was compared with plasma $C\bar{1}$ INH, the purified $C\bar{1}$ INH was diluted in totally $C\bar{1}$ INH-immunodeficient plasma.

CELISA for $C\bar{1}$ INH. Plasma and platelet concentrations of $C\bar{1}$ INH were determined by a CELISA for $C\bar{1}$ INH, which was performed as previously reported for high molecular weight kininogen (12). Goat anti-human $C\bar{1}$ INH was the primary antibody and rabbit anti-goat antibody conjugated with alkaline phosphatase was used as the second antibody. The CELISA assay was usually performed with pooled normal human plasma as the standard. The concentration of $C\bar{1}$ INH in the pooled normal plasmas was determined by standardization with purified $C\bar{1}$ INH. Purified $C\bar{1}$ INH was diluted in totally $C\bar{1}$ INH-immunodeficient plasma according to its protein concentration as determined by the Scopes protein assay (34). The amounts of $C\bar{1}$ INH antigen in two pools of normal human plasma (lots 120 and N10) were then compared with the purified $C\bar{1}$ INH diluted in totally $C\bar{1}$ INH-immunodeficient plasma by RID with anti-$C\bar{1}$ INH antisera. All subsequent dilutions of pooled normal plasma were based on the amount of $C\bar{1}$ INH antigen determined in the pools.

By the CELISA, samples for the standard curve (purified $C\bar{1}$ INH, plasma, and unknowns [plasma, solubilized platelets]) were assayed at 10 different dilutions in triplicate. All data were analyzed on a TRS-80 model III or IV computer (Tandy Corp., Radio Shack Div., Fort Worth, TX). Raw optical density readings were averaged after the blanks were subtracted. Outliers were defined as points with a >10% SD. A best-fit standard curve was determined by an iterative computer program that uses a four-parameter logistic function for a sigmoid line fit of the standard curve (38). This program yields a semilogarithmic graph in which the ordinate is the optical absorbance calculated in relative values from 0 to 1, and the abscissa is the absolute amount of antigen incubated with antisera. Unknown samples were determined by linear least squares regression from the standard curve of the iterative program, and the calculated slope of the regression line was an estimate of the specific activity of the sample (38). Test-sample determinations that were considered valid were computed results that fell on the linear portion of the standard curve for the individual assay and were characterized by a regression analysis with $P < 0.05$ by an F test on the determination of the line and by a t test against the null hypothesis that the slope of the line equals zero. Immunochemical identity of the slope of the linear portions of the standard curves of purified, plasma, and platelet $C\bar{1}$ INH was determined by two-tailed paired t testing and a modified linear regression analysis of Youden (39) where the null hypothesis states that the slope of the line determined equals one.

Preparation of washed platelets. Fresh platelets were washed by a combination of albumin density gradient centrifugation and gel filtration as previously reported (12). In the determinations of all fresh platelet samples for supernatant and total $C\bar{1}$ INH values, blood for platelet-rich plasma was collected into anticoagulant that contained prostaglandin

E_1 (final concentration 1 μM), and these same platelets were gel filtered with buffer that also contained prostaglandin E_1 at the same concentration. Total platelet C$\bar{1}$ INH antigen was determined on washed platelets solubilized with 0.5% Triton X-100 for 30 min at 23°C. In independent studies, the presence of Triton did not interfere with the antigen determination of plasma C$\bar{1}$ INH.

Functional activity of platelet C$\bar{1}$ INH. Glycoprotein extracts of platelets were prepared as previously reported (40) from platelet-rich plasma. Platelets (1.14×10^{10} platelets) from platelet-rich plasma were separated from plasma by centrifugation, gel-filtration over Sepharose 2B, and recentrifugation. After solubilization of the platelet pellet with 0.2% Triton X-100, the supernatant was placed on a Concanavalin A-Sepharose column in order to obtain a partially purified platelet extract. The eluate after introduction of 1 M alpha-methylmannoside was collected and concentrated to 1/1,000 of the starting volume of platelet-rich plasma. The amount of plasma C$\bar{1}$ INH contaminating the platelet extract was estimated by determination of the recovery of ^{125}I-C$\bar{1}$ INH added to the starting platelet-rich plasma in the final material.

Functional C$\bar{1}$ INH activity in the platelet glycoprotein extract was determined by the ability of the partially purified platelet material to neutralize the amidolytic activity of purified kallikrein (27). 60 μl of platelet extract, pretreated for 2 h at 23°C with methylamine to inactivate alpha-2-macroglobulin (four parts platelet extract to one part 0.2 M methylamine, pH 7.4 [27]) was incubated with 0.3 μg of purified kallikrein (7.8×10^{-8} M) (27). At precise intervals (5, 15, 25, 35 min), 10 μl of the mixture was removed and the residual kallikrein amidolytic was determined as previously reported (27). The amount of C$\bar{1}$ INH activity in the platelet extract was calculated by an integrated second-order reaction equation: $(1/INH - K)$ ln $[K(INH - K \cdot INH)/INH(K - K \cdot INH)] = k''T$ (41), where T is the time of the incubation, k'' is the second-order rate constant for C$\bar{1}$ INH inhibition of kallikrein (1.7×10^4 M^{-1} s^{-1}) (42), K is the concentration of kallikrein, and $K \cdot INH$ is the concentration of kallikrein lost by complexing with C$\bar{1}$ INH at a given incubation time. The amount of C$\bar{1}$ INH (INH) was calculated iteratively with a TRS-80 model III computer.

Indirect antibody consumption assay to determine platelet-associated C$\bar{1}$ INH. 7.5 ml of fresh washed platelets (1.5×10^9 platelets/ml), pretreated with 1 μM prostaglandin E_1 and prepared by albumin density gradient centrifugation and gel filtration, was incubated for 45 min at 37°C with an equal volume of anti-C$\bar{1}$ INH antibody. The antibody had previously been diluted to twice its optimal titer in 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4 containing 0.05% Tween-20 and a 1:250 dilution of totally C$\bar{1}$ INH-immunodeficient plasma and centrifuged at 100,000 g for 30 min in an ultracentrifuge (model L3-50; Beckman Instruments, Inc., Palo Alto, CA) to remove aggregates. A second 7.5-ml aliquot of identically washed platelets was centrifuged at 12,000 g in an Eppendorf Centrifuge (Brinkmann Instruments, Inc., Westbury, NY), and the supernatant was incubated 1:1 with anti-C$\bar{1}$ INH antibody prepared as described above. After completion of the incubations and a 4-min centrifugation at 12,000 g in the centrifuge, the antibody adsorbed with platelets was compared with that adsorbed with supernatant on the CELISA by use of known amounts of purified C$\bar{1}$ INH antigen in order to determine a standard curve.

Platelet secretion studies. Fresh platelets washed by albumin density gradient centrifugation and gel filtration were divided into four aliquots of from 5 to 7 ml each in 16 × 95 mm flat-bottomed polystyrene tubes (No. 62.492; Sarstedt, Inc., Princeton, NJ) and placed into a 37°C water bath. The sample for total platelet C$\bar{1}$ INH was solubilized by 0.5% Triton X-100 for 45 min at 37°C. The sample for platelet supernatant C$\bar{1}$ INH was obtained by the centrifugation at 12,000 g of an aliquot of platelets for 4 min followed by collection of the supernatant and incubation for 45 min at 37°C. Washed platelets used in agonist studies were incubated for 30 min at 37°C in the absence or presence of the combined metabolic inhibitors antimycin A (15 μg/ml), 2-deoxy-D-glucose (30 mM), and D-gluconic acid δ-lactone (10 mM) (43, 44). At the end of the incubation, the tubes were placed into a 37°C water bath with stirring at 1,200 rpm, and collagen (20 μg/ml) or thrombin (1 U/ml) was added. After 10 min, the agonist-treated samples were centrifuged at 12,000 g for 4 min and the supernatants were collected. At the end of the secretion experiment, 0.1-ml aliquots were removed from the total, supernatant, agonist-treated, and agonist-plus-metabolic inhibitor–treated samples, and mixed with an equal volume of a mixture of one part 77 mM EDTA, pH 7.4 and nine parts ethanol. These samples were later assayed for total adenine nucleotide (ADP and ATP) content according to the method of Holmsen et al. (45). Similarly, 0.2 ml of each sample was obtained for the determination of platelet lactic dehydrogenase loss according to the method of Wroblewski and Ladue (46). Platelet alpha-granule secretion was assessed by measurement of low affinity platelet Factor 4 by the method of Rucinski et al. (47). The remaining material was used to assay for C$\bar{1}$ INH antigen by the CELISA. All samples were stored at −70°C until assay. Each experiment used combined washed platelets from two normal donors. Percentage secretion (or loss) was determined by the ratio of the supernatant of the agonist-treated specimen to the total platelet lysate after the value of the nonactivated platelet supernatant was subtracted from both. All values represent the percentage of the total amount of each constituent found in the platelet. Washed platelets were also used to prepare Triton-insoluble cytoskeletons according to the method of Tuszynski et al. (48).

Platelet subcellular fractionation. Platelet subcellular fractionation was performed according to the technique of Fukami et al. (49). In each preparation, platelet-rich plasma from 450 ml blood was collected, made 5 mM with EDTA, and pelleted by centrifugation at 3,000 g for 10 min at 4°C. The platelet pellet was suspended in 30 mM Hepes, 5 mM EDTA, and 0.15 M NaCl, pH 6.5. After it was washed by centrifugation twice more at 3,000 g for 10 min at 4°C, the pellet was resuspended in 40 ml of the same medium containing 20 μM rotenone, 5 mM 2-deoxy-D-glucose, and 10 mM D-gluconic acid δ-lactone and incubated at 37°C for 20 min. Then, 3 mg nagarse and 20 mg ATP were mixed into the suspension at 37°C. 5 min later, 20 mg soybean trypsin inhibitor was added to neutralize the nagarse, and the platelets were centrifuged at 3,000 g for 10 min at 4°C. The pellet was then resuspended in 25 ml of 0.01 M Hepes, 1 mM EDTA, and 0.25 M sucrose, pH 7.4 and homogenized twice in a French pressure cell (50) at 500 pounds per square inch, with one centrifugation in between the two homogenizations so that only the unbroken cells were subjected to the second homogenization. 5 ml of the combined homogenate was set aside and 20 ml was used for fractionation. Differential centrifugation of the homogenate resulted in the isolation of four fractions: F_1, 1,000 $g \times 22$ min pellet; F_2, 12,000 $g \times 20$ min pellet; F_3, 100,000 $g \times 60$ min pellet; and F_4, the 100,000 g supernate. The granule fraction, F_2, was then divided in half, and one half was applied to a 0.8 to 2.0 M sucrose step gradient in 0.2-M increments (51). After centrifugation at 100,000 g for 60 min, five distinct areas were collected: A, 0.25–1.0 M sucrose; B, 1.0–1.3 M sucrose; C/D, 1.3–1.8 M sucrose; E, 1.8–2.0 M sucrose; and F, a pellet. Before assay, fractions F_4 and A–E of the F_2 were dialyzed against 0.01 M Hepes, 1 mM EDTA, pH 7.4, lyophilized, and resuspended in 5 ml of the dialysis buffer. All fractions were treated with 0.5% Triton X-100 before assay. C$\bar{1}$ INH antigen was assayed by CELISA. β-*N*-acetyl-glycosaminidase was assayed by the technique of Day et al. (52). Fibrinogen was assayed by EID (37). Serotonin was assayed by the technique of Weissbach et al. (53). Only preparations that had a recovery ≥60% for total protein, C$\bar{1}$ INH, fibrinogen, β-*N*-acetyl-glucosaminidase, and serotonin were used in the analysis. The relative specific amounts of each marker in the preparation were calculated from the ratio of the specific amount of the marker in the fraction (amount antigen or activity per milligram total protein) to the specific amount of the marker in the total platelet lysate.

Results

CELISA for C$\bar{1}$ INH. A CELISA was developed to quantitate and characterize directly C$\bar{1}$ INH in platelets. The typical competition inhibition curves for this assay with monospecific

antibody to C1̄ INH are given in Fig. 2. On the CELISA, the amount of measured optical density from hydrolysis of the substrate was inversely proportional to the amount of soluble antigen present in the incubation mixture. In the CELISA, C1̄ INH antigen in normal plasma was immunochemically identical, giving a parallel standard curve in its linear portion with purified C1̄ INH (two-tailed $P > 0.2$ by modified Youden analysis and >0.1 by paired t test) and with purified C1̄ INH reconstituted in total C1̄ INH-immunodeficient plasma (two-tailed $P > 0.3$ by modified Youden analysis and >0.1 by paired t test) (Fig. 2). Two batches of pooled normal plasma were determined to contain 230 and 236 μg/ml C1̄ INH antigen, respectively, by RID comparison with purified C1̄ INH reconstituted into total C1̄ INH-immunodeficient plasma. When pooled normal plasma was used for the standard curve, the CELISA assay typically gave a linear curve from 10 to 150 ng. In a comparison of the CELISA with EID, 10 individual normal plasmas on the CELISA had a mean±SD C1̄ INH antigen concentration of 233±27 μg/ml (range, 202–264 μg/ml), which was not significantly different (two-tailed $P > 0.1$ by paired t test) from the mean value of 256±42 μg/ml (range, 196–318 μg/ml) of the same plasmas determined by EID. The interassay coefficient of variation of a single plasma sample assayed four times over a 1-mo period was 3.3%. Moreover, the presence of complex formation of C1̄ INH with its proteases after plasma activation did not influence the absolute amount of C1̄ INH antigen measured in the plasma by CELISA. When the plasma concentration of C1̄ INH in a fresh sample was compared with that of the same sample that had been activated by freezing and thawing four times, the C1̄ INH antigen concentration was the same in both plasmas.

Characteristics of washed platelets. To estimate the amount of plasma C1̄ INH that might be contaminating the final washed platelets to be used in these studies, tracer studies were performed with ^{125}I-C1̄ INH added to the starting platelet-rich plasma. (Table I shows one representative experiment of three.) ^{125}I-C1̄ INH (1.35 μg with a specific radioactivity of 2 μCi/μg) was introduced into the platelet-rich plasma, incubated for 5 min at 37°C, and traced through the washing procedure. The final amount of the total ^{125}I-C1̄ INH tracer associated with the washed platelets (3.70×10^8 platelets/ml) was equal to 0.229 of the 1,350 ng of the ^{125}I-C1̄ INH added to the platelet-rich plasma, or 0.017% of the original amount of the radiolabel. This finding indicated that for every milliliter of washed platelets, 39 ng of plasma C1̄ INH or, if the ^{125}I-C1̄ INH tracer was in equilibrium with the platelet and other C1̄ INH, 10.5 ng C1̄ INH/10^8 platelets, would be present in the suspending medium if 1 ml plasma contained 230 μg C1̄ INH antigen. The equilibrium of the ^{125}I-C1̄ INH tracer with platelet-rich plasma was assessed in additional experiments in which the ^{125}I-C1̄ INH tracer was incubated with the platelets in plasma for 3 h. In those experiments there was only a 2% increase in the amount of the radiolabel associated with platelets at the longer incubation. In the CELISA, the amount of antigen directly measured was that present in 0.1 ml of washed platelets. Thus, only 3.9 or 10.5 ng/10^8 platelets of the C1̄ INH antigen present in each assay of solubilized platelets could be estimated to be from plasma C1̄ INH contamination.

*Table I. ^{125}I-C1̄ INH Tracer Studies with Washed Platelets**

Platelet preparation	Total radioactivity‡	Recovery§
	nCi	%
Platelet-rich plasma	1923	100
Washed platelets	0.333	0.017
Supernatant washed platelets	0.196	0.01

* 1.35 μg of ^{125}I-C1̄ INH (specific radioactivity 2 μCi/μg) was added to platelet-rich plasma, and the platelets were washed by albumin density gradient centrifugation and gel filtration. The final washed platelets had a concentration of 370,000 platelets/μl. The data presented are from one representative experiment of three.
‡ Amount given represents the total radioactivity present in the material.
§ Determined from the ratio of the total radioactivity in each material to the total radioactivity of the platelet-rich plasma ×100.

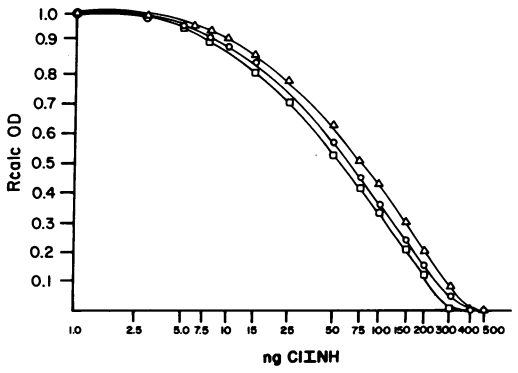

Figure 2. Standard competitive inhibition curve obtained with C1̄ INH antibody on the CELISA. Along the ordinate is the relative absorbance (Rcalc OD), and along the abscissa is the absolute amount of antigen incubated with the antibody. Inhibition curves produced by: △, purified C1̄ INH; □, pooled normal plasma; or ○, total C1̄ INH-immunodeficient plasma reconstituted with purified C1̄ INH.

Determination of platelet-associated plasma C1̄ INH. The amount of plasma C1̄ INH estimated by the ^{125}I-tracer experiment that could contaminate the washed platelets can describe only a lower limit of possible plasma contamination. The presence of soluble, tightly bound, and nonexchangeable plasma C1̄ INH on the platelet surface could possibly account for additional plasma contamination of the washed platelets. To evaluate this possibility an indirect antibody consumption assay was designed to compare antibody adsorbed with platelets with antibody diluted with platelet supernatant on the CELISA. The antibody was diluted in totally C1̄ INH-immunodeficient plasma to provide excess normal immunoglobulin, which prevents nonspecific antibody association with platelets via platelet Fc receptors. C1̄ INH antibody was incubated with whole platelets to determine whether plasma C1̄ INH tightly bound to platelets would adsorb the anti-C1̄ INH antibody, as determined by its ability to detect fixed amounts of purified C1̄ INH. Incubation of platelets under these conditions and centrifugation was associated with <1% lysis as determined by lactic dehydrogenase loss. As shown in Fig. 3, when antibody adsorbed with platelets (1.5×10^9 platelets/ml) was incubated with purified C1̄ INH, a superimposable, parallel standard curve (two-tailed $P > 0.1$ by modified Youden analysis and $P > 0.2$ by paired t test) was produced when compared with

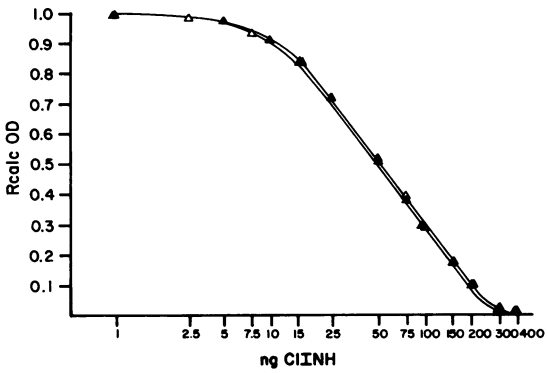

Figure 3. Competitive inhibition standard curve produced by purified C1 INH with antibody adsorbed with platelets and unadsorbed anti-C1 INH antibody. Anti-C1 INH antibody was incubated with equal volumes of whole washed platelets or the supernatant of the platelet suspension. The platelet-adsorbed antibody and unadsorbed antibody were then incubated with equal amounts of purified C1 INH to produce a standard curve by use of the CELISA (Methods). The standard curve produced by platelet-adsorbed antibody (▲) is plotted along with the standard curve produced by unadsorbed antibody (△). In this assay the ordinate expresses relative absorbance (Rcalc OD), and the abscissa shows the absolute amount of antigen incubated with antibody.

purified C1 INH incubated with unadsorbed antibody. This result showed that incubation of whole platelets with anti-C1 INH antibody did not lead to a decrease in the titer of the antibody that could interact with purified C1 INH. This finding indicated that no detectable plasma C1 INH (<6.6 ng/10^8 platelets) was tightly bound to the platelet surface since the standard curve would have been flattened and shifted to the left if the titer of the platelet-adsorbed antibody had been decreased.

C1 INH in washed platelets. Since the ^{125}I-C1 INH tracer studies suggest that 3.9 or 10.5 ng C1 INH antigen/10^8 platelets measured in each aliquot of washed platelets could arise from plasma contamination, the supernatant suspensions of each of the washed platelets from eight donors used in the determination of total platelet C1 INH were directly measured for C1 INH antigen by CELISA. In the eight individual donors, the mean amount of C1 INH antigen directly measured in the platelet supernatants was 7.9±3.2 ng/10^8 platelets (range, 3.7–13 ng/10^8 platelets), indicating close agreement with the value estimated by the ^{125}I-C1 INH tracer experiments. Four of these determinations did not fall within the lower limit of the linear portion of the standard curve, and, therefore, the lowest linear value of the standard curve in the individual assays was used to estimate the amount of supernatant C1 INH antigen. The calculated supernatant value therefore must be an overestimation. The detergent extract of washed platelets from 15 normal platelet donors was studied for total C1 INH antigen (Fig. 4). The amount of platelet C1 INH antigen detected in all extracts had a mean of 62±33 ng/10^8 platelets (mean±SD) with a range of 33–115 ng/10^8 platelets. Supernatant C1 INH antigen comprised 13% of the total platelet C1 INH antigen measured in the eight donors in whom both values were determined, and in no case was it greater than the total value. In four donors with classical hereditary angioedema, the mean platelet C1 INH was 8.3 ng/10^8 platelets with a range of from 5.3 to 11.3 ng/10^8 platelets (Fig. 4). The ability of plasma C1

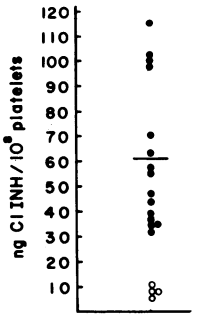

Figure 4. Total platelet C1 INH antigen in platelet lysates. ●, total platelet C1 INH antigen in platelets from normal donors. ○, total platelet C1 INH antigen in platelets from patients with classical hereditary angioedema. All values were determined by CELISA.

INH levels to influence platelet C1 INH values was studied directly by the mixing of washed platelets from a patient with hereditary angioedema with fresh normal plasma. Washed hereditary angioedema platelets with an initial platelet C1 INH antigen concentration of 5.3 ng/10^8 platelets were incubated with fresh normal plasma (C1 INH antigen, 298 μg/ml) for 1 h at 37°C. After the platelets were rewashed, a repeat platelet C1 INH level was found to be 4.8 ng/10^8 platelets. This study indicated that there is little exchange between plasma and platelet pools of C1 INH in unstimulated platelets and that the washing procedure adequately separated the platelets from the plasma. Platelet C1 INH antigen from normal platelets was immunochemically indistinguishable from plasma C1 INH antigen (Fig. 5). This immunochemical identity was indicated by the capacity of C1 INH from solubilized platelets to produce complete competitive inhibition, with an inhibition curve parallel in its linear portion to that produced by plasma (two-tailed $P > 0.4$ by modified Youden analysis and $P > 0.3$ by paired t test). Moreover, when normal platelets were mixed with platelets from a patient with classical hereditary angioedema, the total amount of the antigen present was equal to the sum of platelet C1 INH antigen in each donor.

Functional activity of platelet C1 INH. Glycoprotein extracts of washed platelets neutralized the amidolytic activity of purified kallikrein. Since the extracts were pretreated with methylamine, a chemical inhibitor of alpha-2-macroglobulin (27), the inactivation of kallikrein must have arisen from C1 INH. This C1 INH activity had to arise from platelets since only 2% of the total amount of C1 INH in the platelet extract could be estimated to be from plasma contamination by independent radiolabeled ^{125}I-C1 INH tracer experiments. The concentration of C1 INH in the platelet extract was 9.2±4 × 10^8 M (mean±SD for 15 determinations) for 1.14 × 10^{10} platelets. This value suggested that platelets contain 84 ng functional C1 INH/10^8 platelets (range 48 to 121 ng/10^8 platelets).

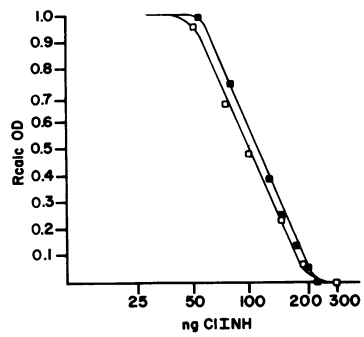

Figure 5. Competition inhibition CELISA comparing the detergent extracts of washed human platelets with plasma C1 INH. Inhibition curves produced by: □, pooled normal plasma; or ■, solubilized platelets. In the particular platelet extract analyzed, the concentration of C1 INH antigen was 56 ng/10^8 platelets.

Secretion of platelet C1̄ INH. Platelet C1̄ INH antigen was secreted from washed platelets by collagen and thrombin (Table II). At a high dose of each agonist, collagen (20 μg/ml) induced secretion of 25% of the total platelet C1̄ INH antigen, and thrombin (1 U/ml) induced secretion of 31% of the total platelet C1̄ INH antigen. This amount of the total antigen secreted for each agonist was less than that seen for a platelet dense granule marker—total adenine nucleotides—and a platelet alpha-granule marker—low affinity platelet Factor 4. With collagen, 45% of the total adenine nucleotides and 52% of the total low affinity platelet Factor 4 were secreted. With thrombin, 68% of both markers was secreted. The amount of platelet C1̄ INH secreted from platelets during the experiments could not be accounted for by cell lysis since collagen resulted in 1.3% of platelet lactic dehydrogenase loss and thrombin accounted for none. The use of metabolic inhibitors showed that platelet C1̄ INH secretion was an active process. At the dose of the agonists used, metabolic inhibitors blocked ≥97% of the secretion of each of the platelet constituents. In two experiments, secreted platelet C1̄ INH antigen did not become part of the Triton-insoluble cytoskeleton of thrombin-stimulated platelets. This finding also indicates that Triton completely solubilizes the platelet C1̄ INH.

Platelet subcellular fractionation. Six individual platelet donors were evaluated for platelet C1̄ INH subcellular localization studies by differential centrifugation. In these six preparations, the mean±SEM recovery for total protein was 78±6%; for C1̄ INH antigen, 81±5%; for fibrinogen antigen, 81±5%; and for β-*N*-acetyl-glucosaminidase activity, 92±4.2%. When washed lysed platelets were subjected to differential centrifugation, the largest amount, as indicated by the area under the graph, of platelet C1̄ INH antigen and of fibrinogen antigen emerged in the 12,000 *g* pellet (fraction F_2), a fraction enriched with platelet granules and mitochondria (Fig. 6).

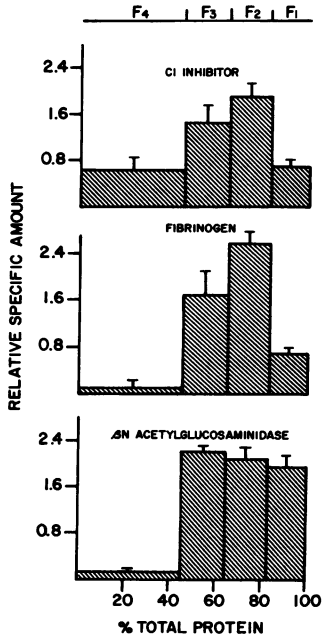

Figure 6. Platelet subcellular fractionation by differential centrifugation. The ordinate shows the relative specific amount of each marker, and the abscissa shows the percentage of total protein in each fraction. The relative specific amount is the ratio of the specific amount in each fraction to the specific amount in the total homogenate of the platelets. The data plotted are the mean±SEM (T) of six experiments.

In an attempt to separate further the components of the granule fraction, the F_2 was subjected to sucrose density ultracentrifugation (Fig. 7). In six experiments, the mean±SEM recovery for total protein was 88±8%; C1̄ INH antigen, 96±14%; fibrinogen antigen, 90±9%; β-*N*-acetyl-glucosaminidase, 77±5%; and serotonin, 98±4.4%. Upon sucrose density ultracentrifugation, 80% of the C1̄ INH antigen from the F_2 fraction entered the gradient (i.e., moved into fractions B through F), indicating that platelet C1̄ INH was contained mostly within platelet granules. Moreover, 81% of granule C1̄ INH antigen (65% of the total C1̄ INH antigen from the F_2

*Table II. Platelet C1̄ INH Secretion: Percentage of Total in Platelets**

	Washed platelets§		Metabolic inhibitor-treated platelets¶	
Markers‡	Collagen‖	Thrombin**	Collagen‖	Thrombin**
C1̄ INH	25±6	31±1.3	0.3±0.3	0
ADP/ATP	45±16	68±11	1.3±1.3	0
LA-PF_4	52±11	68±6.2	0	0.4±0.4
LDH	1.3±1.3	0	0	0

* The platelet agonist was introduced into the platelet suspension and it was stirred at 1,200 rpm at 37°C. The total time from the introduction of the stimulus till the end of stirring was 10 min. Values represent the percentage total secretion, which was determined from the ratio of agonist-treated specimen to the total amount present in detergent lysate after concomitant nonstimulated platelet supernatants were subtracted from both. Values expressed are the means±SEM of three experiments, each consisting of combined platelets from two donors.

‡ ADP/ATP, total adenine nucleotides; LA-PF_4, low affinity-platelet Factor 4; LDH, lactic dehydrogenase. C1̄ INH was assayed by CELISA. See Methods for each other assay procedure.

§ Washed platelets were prepared by albumin density gradient centrifugation and gel filtration (see Methods).

¶ Metabolic inhibitor-treated platelets were platelets incubated with a mixture of antimycin A (15 μg/ml), 2-deoxy-D-glucose (30 mM), and D-gluconic acid δ-lactone (10 mM) for 30 min at 37°C.

‖ Collagen was introduced in the cuvette in a final concentration of 20 μg/ml.

** Thrombin was introduced in the cuvette in a final concentration of 1 U/ml.

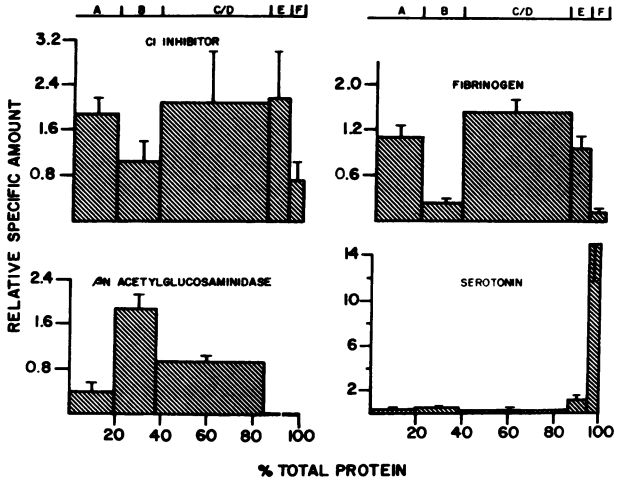

Figure 7. Platelet subcellular fractionation by sucrose density ultracentrifugation of fraction F_2 from Fig. 6. All data are plotted as in Fig. 6.

fraction), as indicated by the area under the graph, was localized to the C/D and E fractions (Fig. 7), fractions previously characterized to be enriched with platelet alpha-granules (48–50). This localization was similar to that of platelet fibrinogen, a recognized platelet alpha-granule constituent (2), but distant from β-*N*-acetyl glucosaminidase, a lysosomal granule constituent; and serotonin, a dense granule constituent. These combined studies suggest that platelet C1̄ INH is mostly a platelet alpha-granule protein.

Discussion

The results from these experiments show that human platelets contained, by immunological criteria, C1̄ INH at an average concentration in normal platelets of 62 ng/10^8 platelets (Fig. 4). In contrast, platelets from patients with classical hereditary angioedema had an average concentration of platelet C1̄ INH of 8.3 ng/10^8 platelets. Two independent approaches, ^{125}I-C1̄ INH tracer studies and direct measurement of C1̄ INH antigen in platelet supernatants from each individual normal donor, estimate that contaminating plasma C1̄ INH could account for only from 7.9 to 10.5 ng/10^8 platelets of the total amount of C1̄ INH antigen measured in the aliquots of each normal platelet lysate as determined by the CELISA. Therefore, plasma contamination could contribute at most only 16% of the C1̄ INH detected in solubilized platelets. The measured value for platelet C1̄ INH on the CELISA appears to represent intracellular platelet C1̄ INH, since C1̄ INH antigen is not detected (<6.6 ng/10^8 platelets) on the unstimulated platelet surface in an indirect antibody consumption assay (Fig. 3). Furthermore, the two pools of C1̄ INH, plasma and platelet, were not exchangeable, since washed platelets from a patient with hereditary angioedema did not increase their C1̄ INH levels after incubation in normal plasma. Finally, platelet C1̄ INH functions like plasma C1̄ INH since it can neutralize the amidolytic of purified kallikrein. Upon functional assay, normal platelets are shown to contain amounts of C1̄ INH activity similar to those determined by antigen studies.

The finding that platelet C1̄ INH is secreted upon stimulation by collagen and thrombin and that this secretion is an energy-requiring process without cell lysis provides a mechanism by which this protein can be made available since it is not found on the surface of unstimulated platelets. The finding that only 25 to 31% of the total amount of platelet C1̄ INH is detected in the suspending medium after platelet stimulation with high agonist concentration needs clarification. There are three possible explanations for this finding. High molecular weight plasma proteins associated with platelet granules may be secreted differently than low molecular weight platelet-specific proteins such as low affinity platelet Factor 4 (47). A higher percentage of this protein may have been secreted but a large portion of it rapidly rebound to the platelet surface. Finally, only a small percentage of the total granule content of this protein was secreted, the rest remaining in granules that became expressed on the activated platelet surface. The two latter mechanisms could provide a means whereby platelet C1̄ INH is expressed in high concentrations on the platelet surface. However, platelet C1̄ INH was not detectable on Triton-insoluble, thrombin-stimulated platelet cytoskeletons prepared by one technique (48), suggesting that it does not become a cytoskeletal component after platelet activation. The secretion of only 25 to 40% of the total platelet content of a high molecular weight hemostatic cofactor contained within platelet alpha-granules, in contrast to low affinity platelet Factor 4 (47), has been noted previously for platelet fibronectin (54), high molecular weight kininogen (12, 55), and von Willebrand factor (11).

Platelet subcellular fractionation studies indicate that 80% of platelet C1̄ INH in the unstimulated platelet is found in those granule fractions that contain alpha-granules (Fig. 7). The relative absence of any significant amount of the protein in the F pellet on the sucrose gradient (Fig. 7), the area where platelet serotonin is largely concentrated, excludes the possibility that platelet C1̄ INH is a dense granule protein. Although 20% of platelet C1̄ INH from the F_2 fraction was found in the A fraction (soluble portion) of the sucrose gradient (Fig. 7), the presence of an equally large amount of platelet fibrinogen in this fraction suggests that some granules may have been lysed in resuspension of the F_2 pellet (Fig. 6) and transfer of it to the gradient for ultracentrifugation. Further confirmation of a platelet alpha-granule localization for platelet C1̄ INH in the unstimulated platelet requires studies on the total content of platelet C1̄ INH in gray platelets (56).

The platelet C1̄ INH level for 3×10^8 platelets/ml plasma is 183 ng, which constitutes 0.08% of the plasma level of 230 μg/ml. This value is similar to that which has been determined for platelet alpha-2-macroglobulin (0.04%) (13), alpha-1-antitrypsin (0.007%) (13, 14), and alpha-2-antiplasmin (0.03%) (20). However, since platelets are concentrated within the fibrin clot, local concentrations of secreted platelet C1̄ INH may exceed physiologic plasma amounts. If platelets secrete their granules by exocytosis and platelet granules are about one-tenth of the volume of platelets (the volume of 10^{11} platelets is 0.7 ml [57]), the concentration of platelet C1̄ INH in granules at the platelet surface during secretion could be ~2.6 mg/ml for 300,000 platelets/μl, 10 times its concentration in plasma. In contrast, activated platelets from the patients with hereditary angioedema could only achieve levels of C1̄ INH similar to the concentration of C1̄ INH in normal plasma. This interpretation enhances the physiologic potential of platelet C1̄ INH, since localized concentrations of the magnitude achievable by normal platelets may effectively promote inhibition of contact-phase proteases (22–25). Recent studies on platelet high molecular weight kininogen indicate that at least a portion equal to that secreted into the suspending medium also becomes expressed on the activated platelet surface (55). This finding suggests that high local concentrations of platelet forms of plasma proteins may be achieved on the activated platelet surface. The finding that platelets contain both the major cofactor (high molecular weight kininogen) (12) and inhibitor (C1̄ INH) to contact phase activation, without containing the protease (kallikrein) (unpublished data) suggests that the platelet locus may be an important site of modulation of surface-activated defense mechanisms involved in complement activation (58, 59) and early blood coagulation.

Note added in proof. We recently studied platelets from a patient with the gray platelet syndrome (patient R. K. (5b), courtesy of Dr. J. G. White, University of Minnesota). The total platelet C1̄INH content in these platelets was 26 ng/10^8 platelets (42% of normal).

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