Properdin Factor D̄

EFFECTS ON THROMBIN-INDUCED PLATELET AGGREGATION

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ABSTRACT Factor D̄, when preincubated with platelet suspensions, at concentrations as low as 1.2 μg/ml, inhibited thrombin-induced platelet aggregation. No inhibition of collagen or arachidonic acid-induced platelet aggregation was found. Inhibition occurred, but to a lesser extent, when thrombin and factor D̄ were added to platelets at the same time. No inhibition occurred when factor D̄ was added after thrombin. Thrombin was able to overcome inhibition by factor D̄ by increasing its concentration. Diisopropylphosphorofluoridate-inactivated factor D̄ also inhibited thrombin-induced platelet aggregation so that enzymatic activity of factor D̄ was not required for inhibition. Factor D̄ absorbed with hirudin coupled to Sepharose 6B showed no decrease in inhibitory capacity. ^{125}I-Factor D̄ bound to platelets in a manner suggesting an equilibrium reaction similar to thrombin. At low factor D̄ input, binding was linear, whereas at higher input, binding began to approach saturation. Binding of ^{125}I-labeled thrombin to platelets was inhibited by factor D̄. Analysis of these data show that factor D̄ does not alter the total number of thrombin molecules which bind to the platelet surface at saturation. However, the dissociation constant for thrombin is altered from 2.78 to 6.90 nM in the presence of factor D̄ (20 μg/ml). Factor D̄ is thus a competitive inhibitor of thrombin binding, although the affiinity of factor D̄ for the platelet thrombin receptor is much less than that of thrombin. These phenomena occur at physiologic concentrations of factor D̄. Therefore, factor D̄ may function in vivo as an inhibitor of platelet aggregation.

INTRODUCTION

Human platelets may interact with proteins of the complement system in several different ways. Anti-platelet antibodies and immune complexes activate platelets partially via classical complement pathway activation (1–3). Zymosan treated with normal human plasma stimulates platelets to aggregate and release serotonin (4, 5). Complement components (deposited on zymosan via alternative pathway activation), fibrinogen, and immunoglobulin (Ig)G are all required for this stimulation of platelets (5–8). Platelets incubated in normal human serum have been found to directly activate and bind C3 and the C5b-C9 complex (9), and to generate C5a chemotactic activity (10). Polley and Nachman have described C3 and C5 activation and binding to platelets in the presence of thrombin, in addition to enhancement of thrombin-induced platelet aggregation and release by C3-C9 (11). The evidence presented in these studies suggested that C3 and C5 cleavage was not mediated by either the classical or alternative pathway convertases.

Thrombin is a potent physiologic activator of human platelets. Activation results in platelet shape changes, aggregation, and release of intracellular biologically active materials. Thrombin must bind to the platelet surface and must have proteolytic activity to induce platelet activation (12–15). The active proteolytic site is not, however, required for binding (12). Present evidence indicates that binding occurs via specific surface receptors with different affinities for thrombin or via a single class of receptors which exhibits negative cooperativity (12, 14–16). Other proteases which have been examined for activation of platelets include trypsin, chymotrypsin, plasmin, and papain (17). Among these, only trypsin and papain were found to induce platelet secretion of calcium, and then only at relatively high concentrations.

Properdin factor D̄ is a 25,000-dalton serine protease which is required for activation of the alternative complement pathway (18–21). Factor D̄ produces limited proteolysis of its natural substrate, properdin factor B, in the presence of C3b, to yield the cleavage fragments Ba and Bb (18–22). The larger fragment, Bb, and C3b form the alternative pathway convertase,

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$\overline{C3bBb}$ (19, 20, 23). Thrombin, but not trypsin or plasmin, may substitute for factor D̄ in a hemolytic assay utilizing factor D̄-depleted serum and guinea pig erythrocytes (24). In addition, thrombin and factor D̄ appear to share antigenic determinants (24).

To develop an approach toward understanding platelet-complement interactions, it is first necessary to know whether individual complement components (particularly those with proteolytic activity) have direct effects upon platelets. Although its zymogen has been described, factor D̄ is apparently present in plasma in its active proteolytic form (21, 25). Platelets therefore may be exposed to active factor D̄. Because it is required for normal activation of the alternative pathway, any interactions between complement and platelets should take into account possible influences by factor D̄. We have therefore examined the effects of highly purified factor D̄ upon platelet aggregation and upon thrombin binding to platelets.

METHODS

Platelet preparation. Human blood obtained by the American Red Cross Blood Services, Northeast Region, from male volunteers who denied ingesting aspirin for 1 wk before donation, was collected by free flow into acid citrate dextrose, NIH formula A at a ratio of 1.67/10 ml whole blood. Whole blood was centrifuged at 230 *g* for 15 min to obtain platelet-rich plasma. For most experiments, washed platelet suspensions were prepared by adding 0.2 vol acid citrate dextrose to platelet-rich plasma which was then centrifuged at 800 *g* for 15 min to pellet the platelets. Platelets were resuspended in a modified Tyrode's solution (26) of 136 mM NaCl, 2.6 mM KCl, 0.5 mM NaH_2PO_4, 2 mM $MgCl_2$, 5.5 mM glucose, 0.2% human serum albumin, and to substitute for bicarbonate 10 mM *N*-tris(hydroxymethyl)-methyl-2-amino ethane sulfonic acid (TES)[1] at pH 7.35 (TES/Tyrode's) to which 0.1 vol acid citrate dextrose was added. After centrifugation at 800 *g* for 12 min, the pellets of once washed platelets were resuspended in the above TES/Tyrode's buffer without acid citrate dextrose. For some experiments, gel-filtered platelets were prepared by the technique of Lages et al. (27). For thrombin and factor D̄ binding, platelets were washed twice in a solution of 144 mM NaCl, 10 mM TES, pH 7.3, (TES-saline) with 5.5 mM glucose. Platelets were then resuspended in 140 mM cacodylate, buffered at pH 7.35 with 15 mM TES, and containing 5.5 mM glucose as suggested by Shuman and Majerus (28) who showed that chloride inhibits and cacodylate optimizes thrombin binding to platelets. All platelet separations were at room temperature and platelets were counted electronically with a Coulter counter (model B, Coulter Electronics Inc., Hialeah, Fla.).

Platelet aggregation. Assays for platelet aggregation were performed on a platelet aggregometer (Chrono-Log Corp., Havertown, Pa.). In most experiments, washed platelets in TES/Tyrode's at 200,000 cells/μl and, TES/Tyrode's alone were used to calibrate 5 and 95% light transmission, respectively. For some experiments, to increase the sensitivity of the aggregation assay and measure response to low levels of thrombin, 5% light transmission was adjusted to the turbidity of 200,000 platelets/μl and 95% light transmission to the turbidity of 100,000 platelets/μl.

To study effects of factor D̄ on platelet aggregation, platelets in TES/Tyrode's at 250,000 cells/μl were warmed to 37°C for 5 min. Purified factor D̄, in 144 mM NaCl, 10 mM TES, pH 7.35, was added to the platelets to give a final platelet concentration of 200,000 cells/μl. Incubations of platelets with factor D̄ were carried out as specified in Results.

All reagents were added to platelet suspensions in volumes of 10 μl or less to give final concentrations indicated in the figure legends. Bovine topical thrombin (Parke, Davis & Company, Detroit, Mich.) was dissolved in 150 mM NaCl at 500 U/ml, stored at −20°C, and standardized against NIH standard thrombin (lot B3) before use. Highly purified human thrombin (1–2 U/μg) was prepared and supplied by Dr. John Fenton, II, New York State Department of Health, Albany, N. Y., and by Dr. Robert Rosenberg, Dana Cancer Research Center, Children's Hospital Medical Center, Boston, Mass. Every aggregation experiment reported was performed at least five times with bovine thrombin, except the inhibition of aggregation by diisopropylphosphorofluoridate (DFP)-treated factor D̄ and thrombin which were done with platelets from one donor. Collagen (bovine tendon) was obtained as a suspension of 1 mg/ml in acetate buffer from Hormon-Chemie, Munich, Germany and arachidonic acid, obtained as the sodium salt from Nu-Chek Prep, Elysian, Minn., was dissolved in 100 mM $NaCO_3$; equivalent concentrations of solvents for collagen and arachidonic acid were added to controls.

Binding experiments. Factor D̄ was labeled with ^{125}I by the technique of Bolton and Hunter (29). Incorporation of ^{125}I was 0.7 μCi/μg. There was no change in factor D̄ functional activity after labeling. Purified human thrombin was also labeled with ^{125}I by the technique of Bolton and Hunter (29). Protein-bound ^{125}I was separated from free Bolton-Hunter reagent by gel filtration on Sephadex G-25. Incorporation of ^{125}I was 5.2 μCi/μg. Specific activity of the ^{125}I-thrombin was 1.0 U/μg. Binding of ^{125}I-factor D̄ and ^{125}I-thrombin by washed platelets were measured by a modification of the technique of Martin et al. (14). Suspensions of washed platelets in a solution of 140 mM cacodylate, 15 mM TES, and 5.5 mM glucose, pH 7.35, contained 2.5×10^8 platelets/ml and were used within 1 h of preparation. Binding experiments were at room temperature in a total volume of 325 μl containing platelets and thrombin and(or) factor D̄. Platelets and thrombin were incubated at room temperature for 1 min; platelets and factor D̄ were incubated for 10 min. Platelets were separated from the suspending medium by centrifugation through a 0.1-ml layer of silicone oil (Versilube F-50, Hartwig Chemical Co.) for 45 s at 7,000 *g* in a semimicrofuge (model 59, Fisher Scientific Co., Pittsburgh, Pa.). After centrifugation, duplicate samples of the aqueous phase above the oil were transferred to counting vials, and the plastic tubes were cut at the oil layer just above the platelet pellets. ^{125}I-Factor D̄ and ^{125}I-thrombin were counted directly in supernates and pellets with a γ-counter (model 1185, Searle Diagnostics Inc., subsidary of G. D. Searle & Co., Des Plaines, Ill.). The extracellular space accessible for nonspecific thrombin and factor D̄ trapping was estimated with [^{14}C]inulin (New England Nuclear, Boston, Mass.) at 2.5 mCi/mM. Factor D̄ did not alter inulin trapping compared to controls. At the concentrations of factor D̄ and thrombin used, inulin trapping was negligible (<0.01%) and values for binding shown in Figs. 4 and 5 were not corrected for trapping. Nonspecific binding was determined by incubation of the various concentrations of ^{125}I-thrombin

[1] *Abbreviations used in this paper*: DFP, diisopropylphosphorofluoridate; K_{diss}, dissociation constant; TES, *N*-tris-(hydroxymethyl)methyl-2-amino ethane sulfonic acid.

(0.01–0.1 U/ml) in the presence of 5 U/ml unlabeled purified bovine thrombin (Sigma Chemical Co., St. Louis, Mo.), as described by Tollefsen et al. (12). Nonspecific binding varied from 20 to 30%. Values for thrombin binding plotted in Fig. 5 were obtained by subtraction of the amount of thrombin bound nonspecifically from the amount bound either in the absence or presence of factor D̄.

Properdin factor D̄ purification. Two methods were used for factor D̄ purification. Initial experiments were performed with factor D̄ isolated as described by Volanakis et al. (22). Subsequent experiments were performed with factor D̄ isolated by the following method.[2] 4 liters of fresh, frozen plasma obtained from the American Red Cross Blood Services, Northeast Region, were applied to a 2-liter column of CM Sephadex C-50 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) equilibrated in 0.05 M sodium phosphate, 0.01 M disodium EDTA, pH 6.0. Factor D̄ was eluted with a 4-liter linear gradient from 0.3 to 2.0 M NaCl in the above buffer. After concentration by ultrafiltration on an Amicon PM-10 membrane (Amicon Corp., Lexington, Mass.), the factor D̄-containing pool was applied to an 8 × 90-cm Sephadex G-75 column equilibrated in 0.05 M barbital buffer containing 0.3 M NaCl and 0.01 M EDTA, pH 7.4. Final purification was obtained by further gel filtration on 2.5 × 90-cm Sephadex G-75 columns. On 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (30), purified factor D̄ (20 μg) shows >90% of the stainable protein contained within a single band (Fig. 1). Molecular weight as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis was 25,000. Purified factor D̄ gave a single stainable protein band with an isoelectric point of pH 7.0 after isoelectric focusing (31). This band contained factor D̄ functional activity as determined by the hemolysis overlay technique of Martin et al. (32). Factor D̄ functional activity was confirmed by demonstration of cleavage of properdin factor B by crossed immunoelectrophoresis after incubation with factor D̄ and cobra venom factor. Neither immunochemical nor functional C3 or factor B could be demonstrated in factor D̄ preparations. No fibrinogen-clotting activity could be detected in purified factor D̄ preparations. Factor D̄ (10 μg/ml), when incubated for as long as 24 h with either pooled plasma or with purified fibrinogen, did not induce clot formation.

Affinity chromatography. 220 U Hirudin (Pentapharm AG, Basel, Switzerland) (1 U/μg) was covalently linked to 2 ml cyanogen bromide-activated Sepharose 6B (Pharmacia Fine Chemicals) by the method of Parikh et al. (33). Samples containing thrombin, factor D̄, or buffer alone were incubated for 1 h at 22°C with hirudin-Sepharose, and were then centrifuged at 100 *g* for 5 min. Supernates were removed and assayed for clotting and platelet-aggregating activity. Heparin (2.67 mg/ml Sepharose) was linked to cyanogen bromide-activated Sepharose 6B by the same technique (34).

RESULTS

Inhibition of platelet aggregation with factor D̄. Factor D̄ concentrations ranging from 1.2 to 9.0 μg/ml were preincubated with platelet suspensions for 5 min at 22°C. As shown in Fig. 2A, factor D̄ produced a definite decrease in the rate and extent of thrombin-induced platelet aggregation of washed platelets, but had no effect upon platelet aggregation induced by either arachidonic acid (80 μM) or collagen

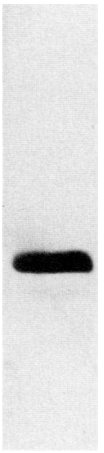

FIGURE 1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified factor D̄. No reducing agents were added to the 20 μg of purified factor D̄ applied to the gel.

(Fig. 2B). Factor D̄ did not induce platelet lysis, as indicated by the lack of lactate dehydrogenase activity in supernates (35). Factor D̄ inhibited thrombin-induced aggregation of both washed and gel-filtered platelets with equal efficiency. Platelets from different donors did show some variability both in response to thrombin and to inhibition by factor D̄. Inhibition of thrombin-induced platelet aggregation was confirmed with each of 15 platelet preparations from different donors and with five different highly purified factor D̄ preparations. The results shown in Fig. 2 are typical of these experiments.

Thrombin-factor D̄ competition. Table I depicts the effects of different quantities of factor D̄ added to platelet suspensions at different times. Inhibition of aggregation was maximal when platelets were preincubated with factor D̄ for 5 min before addition of thrombin. Inhibition occurred, but to a lesser extent, when thrombin and factor D̄ were added simultaneously to the platelet suspension. No inhibition occurred if factor D̄ was added after thrombin (data not shown). In other experiments, thrombin and factor D̄ were combined and incubated for 5 min before addition to platelet suspensions. The degree of inhibition was the same as in the experiments in which thrombin and factor D̄ were added separately but at the same time. Increasing quantities of thrombin were added to platelets which had been preincubated for 5 min with factor D̄. As shown in Table I, additional thrombin was able to partially overcome the inhibition and induce a platelet response. Whereas 0.07 U/ml thrombin produced maximal aggregation in the absence of factor D̄, 0.17 U/ml was required with factor D̄ present.

Effect of inactivation of factor D̄ with DFP. 36 μg factor D̄ and 300 U thrombin in 0.2 ml TES-saline were each inactivated by the addition of 0.1 M DFP

[2] Davis, A. E., III. Manuscript in preparation.

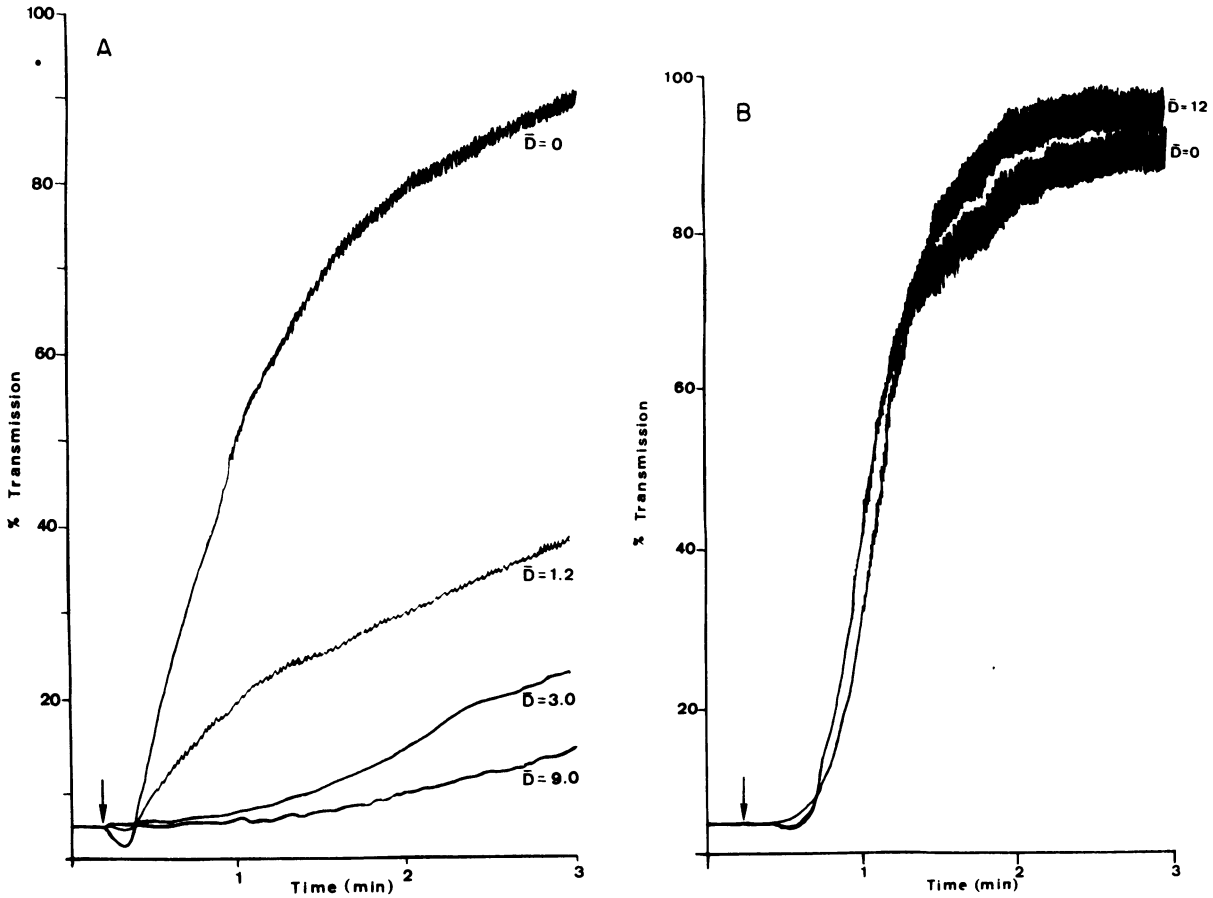

FIGURE 2 (A) Effect of factor D̄ on platelet aggregation induced by thrombin. Purified human thrombin at 0.1 U/ml was added as indicated by the arrow. Platelet suspensions were preincubated with factor D̄, concentrations in micrograms per milliliter, for 5 min at 37°C. (B) Collagen-induced aggregation of platelets in the presence of factor D̄. Collagen at 3 μg/ml was added as indicated by the arrow. Platelets were preincubated with factor D̄ (12 μg/ml) for 5 min at 37°C.

in propylene glycol to a final concentration of 0.001 M. Buffer control consisted of 0.1 M DFP added to 0.2 ml TES-saline to a final concentration of 0.001 M. Samples were maintained at 0°C for 2 h before use in aggregation experiments. Both the factor D̄ and the thrombin were >90% inactivated by hemolytic assay and by fibrinogen-clotting activity respectively. The buffer control had no effect upon thrombin-induced platelet aggregation. DFP-thrombin binds to platelets in a manner indistinguishable from proteolytically active thrombin (12). 8.6 U/ml DFP-thrombin inhibited aggregation by 80% at 2.5 min, at which time control aggregation with 0.1 U/ml thrombin was complete (Fig. 3). 9 μg DFP-factor D̄ inhibited aggregation by 70% at 2.5 min. This single experiment suggests that proteolytic activity is not required for inhibition. These and the previous data suggest that factor D̄ competes with thrombin for the same membrane-binding sites.

The effect of hirudin upon the inhibition of aggregation. 1 U thrombin and 18 μg factor D̄ were each absorbed with 0.25 ml (27.6 U) settled hirudin-Sepharose. Thrombin absorbed with hirudin-Sepharose did not clot fibrinogen and did not induce platelet aggregation. Under the conditions used for these absorptions, the derivative thrombins produced by trypsin digestion or autolysis, are removed as efficiently as α-thrombin (data not shown). Buffer alone incubated with hirudin-Sepharose had no effect upon thrombin-induced platelet aggregation or upon the ability of thrombin to clot fibrinogen. Hirudin itself was therefore not being eluted from the Sepharose during incubation. Factor D̄ absorbed with hirudin-Sepharose showed no loss in factor D̄ hemolytic activity, as has been previously reported (36). There was no loss in the ability of factor D̄ (9 μg) to inhibit thrombin-induced platelet aggregation after absorption. It thus appears unlikely that inhibition is due to contamination of factor D̄

TABLE I
Thrombin-Induced Platelet Aggregation in the Presence of Factor D̄

Thrombin*	Factor D̄	Extent of aggregation	Lag time
U/ml	*μg/ml*	*% control at 3 min*	*min*
(A)			
0.03	0	100	0.25
0.03	6—Preincubated	54	1.10
0.03	12—Preincubated	15	2.00
0.03	6—No preincubation	97	0.35
0.03	12—No preincubation	23	0.60
(B)			
0.07	0	100	0.20
0.07	6—Preincubated	36	1.40
0.12	0	100	0.20
0.12	6—Preincubated	71	1.25
0.17	0	100	0.20
0.17	6—Preincubated	86	0.75

* Bovine thrombin.
(A) The effect of preincubation of platelets with factor D̄ for 5 min at 37°C.
(B) The effect of increasing thrombin concentrations. Inhibition of aggregation was quantitated by comparing the total increase in light transmission at 3 min, at which time aggregation in controls was complete (extent of aggregation), and the time between addition of thrombin and the onset of aggregation (lag time).

preparations with thrombin fragments which might be capable of binding to platelets. Occasional factor D̄ preparations are contaminated with a protein with a molecular weight of ≅15,000 (very faintly staining lower band in Fig. 1). This contaminant was separated from factor D̄ by chromatography on heparin-Sepharose equilibrated in 0.05 M imidazole-HCl, pH 6.0, 0.0025 M $CaCl_2$. Under these conditions, factor D̄ binds to heparin-Sepharose, and the lower molecular weight protein does not. The contaminating protein, at a concentration of 20 μg/ml did not inhibit thrombin-induced platelet aggregation. Factor D̄, eluted from the heparin-Sepharose with 0.5 M NaCl, retained its full inhibitory activity.

Binding of ^{125}I-factor D̄ to platelets. Factor D̄ radiolabeled with ^{125}I was incubated for 10 min with platelet suspensions. Quantities of factor D̄ varied from 0.13 to 27.0 μg/ml. After separation of platelets from medium and determination of bound and free radioactivity as described in Methods, total and platelet-bound factor D̄ were calculated and plotted (Fig. 4). Binding was linear at low factor D̄ input and appeared to approach saturation at higher factor D̄ input. The results shown are representative of three different experiments.

Inhibition of ^{125}I-thrombin binding to platelets.

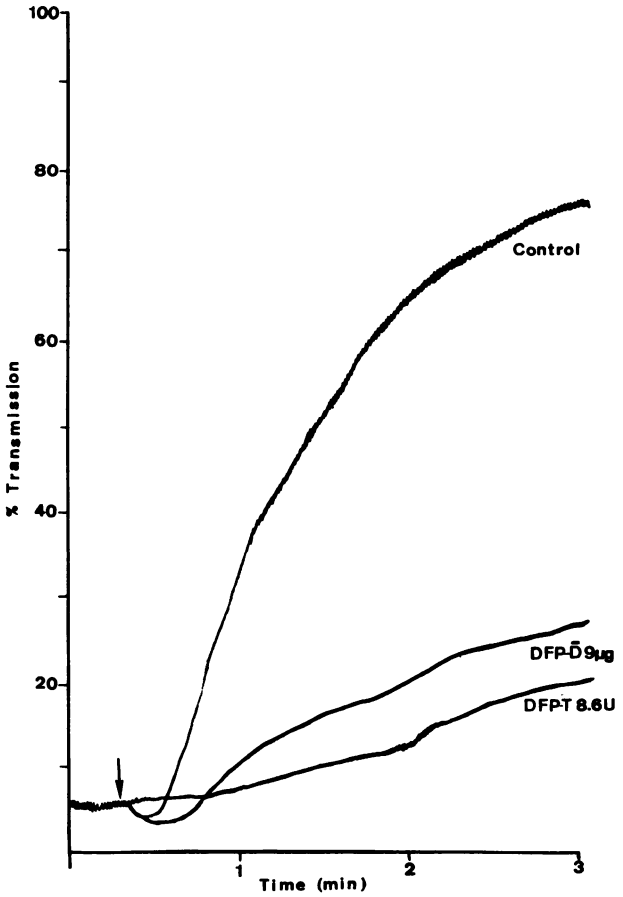

FIGURE 3 Inhibition of thrombin-induced platelet aggregation by DFP-treated factor D̄ and thrombin. A platelet suspension from a single donor was preincubated with DFP-thrombin (DFP-T) and DFP-factor D̄ (DFP-D̄), at concentrations per milliliter, for 5 min at 37°C before the addition of purified human thrombin (0.1 U/ml as indicated by the arrow).

The inhibition of ^{125}I-thrombin binding to platelets is shown in Fig. 5. ^{125}I-Thrombin at concentrations ranging from 0.01 to 0.10 U/ml (0.27–2.7 pmol/ml) was incubated for 1 min with platelet suspensions (2.5×10^8 platelets/ml) which had been preincubated for 10 min at 23°C, either alone or in the presence of 20 μg/ml factor D̄. All incubations were performed in quadruplicate. After incubation, platelets were separated from the suspending medium, and free and bound ^{125}I-thrombin were determined as described in Methods. Fig. 5 depicts one of three experiments, each of which yielded very similar results. Although there were slight differences in total thrombin bound and in nonspecific binding with platelets from different donors, the degree of inhibition of thrombin binding by factor D̄ was identical in the three experiments.

Analysis of the binding data according to the method of Steck and Wallach (37) indicate that factor D̄ is a competitive inhibitor of thrombin binding (Fig. 6).

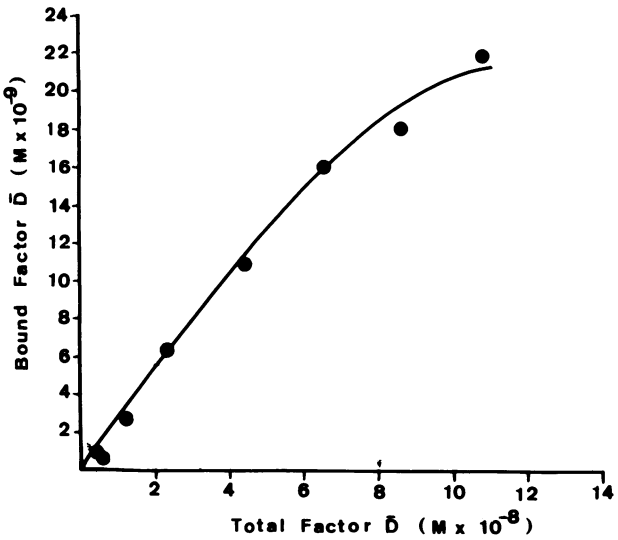

FIGURE 4 ^{125}I-Factor D̄ binding to platelets. Each 10 min incubation was conducted in 0.25 ml of isotonic cacodylate (pH 7.35) with a platelet concentration of 2.5 × 10^8/ml at 22°C. Incubations were terminated by centrifugation of platelets through silicone oil as described in Methods.

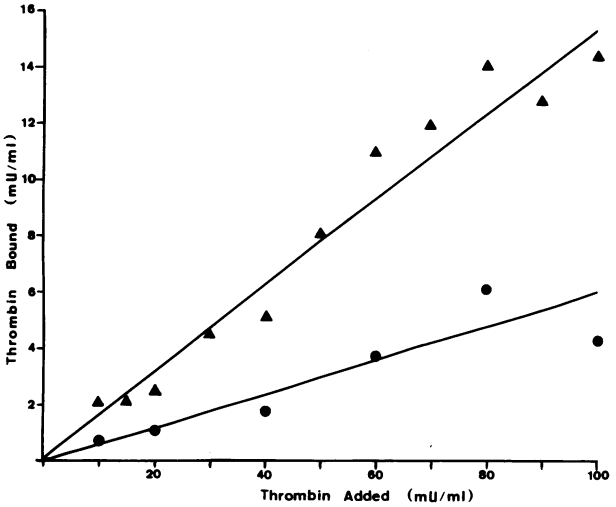

FIGURE 5 Binding of ^{125}I-thrombin to platelets after preincubation in buffer alone and in the presence of factor D̄ (20 μg/ml). Each incubation contained 2.5 × 10^8 platelets/ml and the indicated concentration of thrombin in a final volume of 0.325 ml of isotonic cacodylate, pH 7.35. Incubations with factor D̄ were for 10 min, incubations with thrombin were for 1 min. Platelet separations, and free and bound ^{125}I-thrombin were determined as described in Methods. Plotted points represent the mean values of quadruplicate incubations. The lines were determined by linear regression analysis on a calculator (model 33E, Hewlett-Packard Co., Palo Alto, Calif.) from all data points. (▲) ^{125}I-thrombin bound in the absence of factor D̄ after subtraction of nonspecific binding (determined as described in Methods); (●) ^{125}I-thrombin bound in the presence of factor D̄ after subtraction of nonspecific binding.

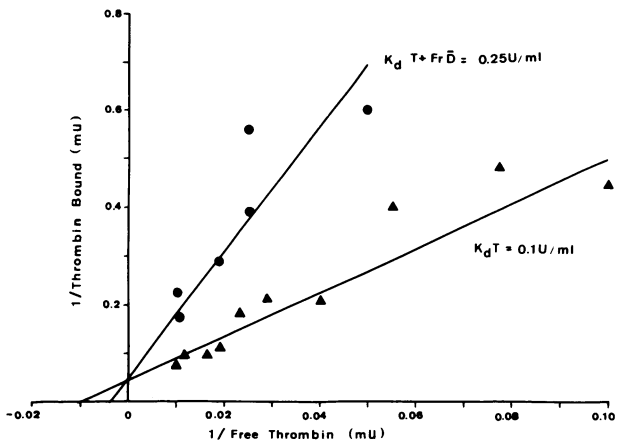

FIGURE 6 Competitive inhibition of ^{125}I-thrombin binding to platelets by factor D̄. The data from Fig. 5 are plotted according to the method of Steck and Wallach (37). The intercept on the ordinate equals 1/thrombin bound at saturation per 2.5 × 10^8 platelets. The intercept on the abscissa equals −1/thrombin free per 1.0 ml (K_{diss}). (▲) ^{125}I-thrombin in the absence of factor D̄; (●) ^{125}I-thrombin in the presence of factor D̄. The lines were determined by linear regression analysis on a Hewlett-Packard model 33E calculator.

Based upon this data, the number of molecules of thrombin bound per platelet, calculated from the intercept on the ordinate, is ≅1,500, either in the absence or presence of factor D̄. The dissociation constant (K_{diss}) for thrombin, calculated from the intercept on the abscissa is 0.10 U/ml (2.78 nM). In the presence of 20 μg/ml factor D̄ (0.8 μM) however, the K_{diss} for thrombin is 0.25 U/ml (6.9 nM). Factor D̄, therefore, is a competitive inhibitor of thrombin binding, although it has a much lower affinity for binding than does thrombin. The inhibiton constant for factor D̄ calculated from this data (38) is 0.53 μM.

DISCUSSION

The data presented in this study demonstrate that properdin factor D̄ specifically inhibits thrombin-induced platelet aggregation. Further, the data suggest that factor D̄ and thrombin compete for the same receptors on the platelet surface. The fact that preincubation of factor D̄ with platelets results in more effective inhibition, and the ability to overcome inhibition with increasing thrombin concentrations are both consistent with this interpretation. In addition, factor D̄ inactivated with DFP inhibits as effectively as does active factor D̄, which indicates that proteolytic activity is not required. Factor D̄ is thus not simply removing thrombin receptors or nonspecifically altering the platelet membrane. If this were the case, not only would proteolytic activity be necessary, but inhibition would not be reversible with increasing thrombin

concentrations. The inhibition of platelet aggregation by factor D̄ is specific for thrombin. No inhibition was observed when other activating agents were used. This indicates that factor D̄ does not simply "paralyze" the platelet's ability to respond, and lends further support to the above hypothesis.

The binding experiments demonstrate that factor D̄ binds to platelets in a manner very similar to thrombin (12, 14, 15). In addition, factor D̄ inhibits binding of ^{125}I-thrombin to platelets. The number of binding sites on the platelet surface calculated from our data is slightly higher than the number reported in some previous studies (12, 14, 15). This is very likely due to the presence of cacodylate in the buffers used in the binding studies reported here, as has been shown previously (28). The total number of thrombin molecules bound at saturation was not changed by factor D̄, whereas the apparent K_{diss} for thrombin was increased, which indicates competitive inhibition. The concentration of factor D̄ used in the thrombin-binding study was similar to the concentrations required to inhibit thrombin-induced platelet aggregation. The alteration of the K_{diss} for thrombin by factor D̄ (and its calculated inhibition constant) suggested that inhibition of thrombin-induced platelet aggregation is secondary to competitive inhibition of thrombin binding to the platelet surface. Factor D̄ has a lower affinity for the platelet thrombin receptor than does thrombin. Factor D̄, at physiologic concentrations, would therefore be expected to interfere with thrombin action upon platelets in situations in which low thrombin concentrations are generated. With generation of high thrombin concentrations, factor D̄ would probably have no effect upon platelet-thrombin interactions.

None of the evidence suggests that the observed inhibition might be a result of contamination of factor D̄ preparations with thrombin fragments. If an undetected thrombin fragment with an intact binding site but without proteolytic activity were present, the observed inhibition might occur. Evidence has been reported which suggests that the binding site on thrombin for hirudin is related to the binding site for platelets (39). Absorption with hirudin-Sepharose would therefore be expected to remove any thrombin fragments which might bind to platelets and inhibit aggregation. Factor D̄ absorbed with hirudin-Sepharose remained fully capable of inhibiting aggregation.

These data provide further evidence that factor D̄ and thrombin are closely related proteases. Thrombin has factor D̄ activity in a hemolytic assay and also apparently shares antigenic determinants with factor D̄ (24). Initial analysis of the amino acid sequence of factor D̄ demonstrates that it is not a fragment of thrombin, which was suggested as one possible explanation for the above findings.[2]

It is not clear whether the phenomenon described here has any direct relationship with previously described complement-platelet interactions. Aside from its role in activation of the alternative pathway by zymosan, it seems unlikely the factor D̄ is directly involved in the activation of platelets by zymosan which has been incubated in plasma (4–8). In addition, no direct data are available to suggest that factor D̄ might play a part in the activation of C3 and terminal complement components by platelets (9, 10), or by platelets in the presence of thrombin (11). The data presented in these studies suggested, in fact, that the alternative pathway was not directly involved in these phenomena. However, the possibility that thrombin or factor D̄ may activate a membrane-bound protease which is capable of activating C3 and C5 remains to be examined.

Although there is no direct evidence to suggest that inhibition of thrombin-induced platelet aggregation by factor D̄ is of physiologic relevance, the amount of factor D̄ required is well within physiologic ranges. The normal serum level of factor D̄, determined by radioimmunoassay, is reported to be ≅2 μg/ml (25). Factor D̄, at concentrations of 1.2 μg/ml was capable of inhibition of aggregation. It is possible, therefore, that factor D̄ may function in vivo as an inhibitor of platelet aggregation.

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