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K A Bauer, ... , B L Kass, R D Rosenberg

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Research Article

The presence of congenital antithrombin deficiency has been consistently shown to predispose patients to venous thrombosis. We have utilized the prothrombin fragment F1+2 radioimmunoassay to quantitate factor Xa activity in the blood of 22 asymptomatic individuals with this clinical disorder not receiving antithrombotic therapy. The mean level of F1+2 was significantly elevated in these patients as compared to normal controls (3.91 vs. 1.97 nM, P less than 0.001). The metabolic behavior of ¹³¹I-F1+2 was found to be similar in antithrombin-deficient subjects and normal individuals. The hemostatic system hyperactivity as measured by the F1+2 assay could be specifically corrected by raising the plasma antithrombin levels of the above asymptomatic individuals into the normal range. This study provides the first demonstration that the prethrombotic state can be biochemically defined as an imbalance between the production and inhibition of factor Xa enzymatic activity within the human circulation. It is known that antithrombin and alpha 1-proteinase inhibitor (PI) are the major inhibitors of factor Xa in human plasma in the absence of heparin. To further evaluate the mechanism by which antithrombin functions as an inhibitor of factor Xa in humans, we studied five patients who exhibited severe congenital deficiencies of alpha 1-PI. Our results indicated that the plasma of these subjects showed virtually identical decreases in plasma antifactor Xa activity in the absence [...]

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Elevated Factor Xa Activity in the Blood of Asymptomatic Patients with Congenital Antithrombin Deficiency

Kenneth A. Bauer, Thomas L. Goodman, Bryon L. Kass, and Robert D. Rosenberg

Charles A. Dana Research Institute and Harvard-Thorndike Laboratory, Department of Medicine, Beth Israel Hospital and Harvard Medical School, Boston 02215; Department of Biology and Whitaker College, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Abstract

The presence of congenital antithrombin deficiency has been consistently shown to predispose patients to venous thrombosis. We have utilized the prothrombin fragment F_{1+2} radioimmunoassay to quantitate factor Xa activity in the blood of 22 asymptomatic individuals with this clinical disorder not receiving antithrombotic therapy. The mean level of F_{1+2} was significantly elevated in these patients as compared to normal controls (3.91 vs. 1.97 nM, $P < 0.001$). The metabolic behavior of ^{125}I - F_{1+2} was found to be similar in antithrombin-deficient subjects and normal individuals. The hemostatic system hyperactivity as measured by the F_{1+2} assay could be specifically corrected by raising the plasma antithrombin levels of the above asymptomatic individuals into the normal range. This study provides the first demonstration that the prethrombotic state can be biochemically defined as an imbalance between the production and inhibition of factor Xa enzymatic activity within the human circulation. It is known that antithrombin and α_1 -proteinase inhibitor (PI) are the major inhibitors of factor Xa in human plasma in the absence of heparin. To further evaluate the mechanism by which antithrombin functions as an inhibitor of factor Xa in humans, we studied five patients who exhibited severe congenital deficiencies of α_1 -PI. Our results indicated that the plasma of these subjects showed virtually identical decreases in plasma antifactor Xa activity in the absence of heparin when compared to antithrombin-deficient individuals, but the plasma F_{1+2} levels in the α_1 -PI deficient population were not significantly different than normal. This data suggests that α_1 -PI does not function as a major inhibitor of factor Xa in vivo, and that a tonically active heparin-dependent mechanism exists in humans for accelerating the neutralization of this enzyme by antithrombin.

Introduction

The conversion of prothrombin to thrombin is the central event in the coagulation of blood. This reaction takes place at an appreciable rate under physiologic conditions only in the presence of factor Xa, factor Va, calcium ions, and platelets. During this process, the amino terminus of the prothrombin molecule is released as the inactive F_{1+2} fragment in conjunction

with the generation of thrombin. Once evolved, this serine protease is able to cleave two pairs of peptides from fibrinogen, fibrinopeptides A and B, which permits the resulting fibrin to polymerize into an insoluble clot. Alternatively, thrombin can be inhibited by its natural antagonist, antithrombin, via the formation of a stable enzyme-inhibitor complex. Although other plasma proteins are able to inactivate this enzyme, antithrombin is the inhibitor of primary physiologic importance (1–3). Other serine proteases of the coagulation system that are neutralized by this inhibitor include factors IXa, Xa, XIa, and XIIa (4–7). Each of these enzyme-inhibitor interactions is accelerated by heparin (1, 4–7).

We have developed a sensitive and specific radioimmunoassay (RIA)¹ for F_{1+2} which measures the in vivo cleavage of the prothrombin molecule by factor Xa (8, 9). Nessel and co-workers (10–12) have established a similar assay for fibrinopeptide A (FPA), which quantitates the in vivo cleavage of fibrinogen by thrombin. For the work reported here, we have utilized these immunochemical techniques to demonstrate that substantial elevations in factor Xa activity, but not thrombin activity, regularly occur in the blood of asymptomatic individuals with congenital antithrombin deficiency, an inherited disorder known to be highly correlated with the subsequent development of thrombosis. This biochemical abnormality could be specifically corrected by raising the plasma antithrombin levels of the above asymptomatic individuals into the normal range. We have also compared our findings in this population with those in a cohort of patients with low levels of α_1 -proteinase inhibitor (PI), a plasma protein which makes a substantial contribution to in vitro plasma antifactor Xa activity in the absence of heparin. Based upon the accumulated data, we suggest that a tonically active heparin-dependent mechanism exists within the vascular system of humans for accelerating the neutralization of factor Xa by antithrombin. Our studies also demonstrate that the prethrombotic state can be biochemically defined by utilizing techniques, such as the F_{1+2} assay, that quantitate the in vivo activity of the coagulation mechanism in the circulation.

Methods

Column chromatographic materials and reagents. DEAE-cellulose and dry hydroxylapatite were purchased from Bio-Rad Laboratories, Richmond, CA. Sephadex G-25 and Heparin-Sepharose CL-6B were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Heparin (Panheprin) was bought from Abbott Laboratories, North Chicago, IL.

Proteins. Bovine serum albumin was purchased from Sigma Chemical Co., St. Louis, MO. Human prothrombin was prepared by the

Address reprint requests to Dr. Bauer, Beth Israel Hospital, 330 Brookline Avenue, Boston, MA 02215.

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1. Abbreviations used in this paper: FPA, fibrinopeptide A; PI, proteinase inhibitor; RIA, radioimmunoassay.

method of Shapiro et al. (13) with minor modifications as described in a prior communication from our laboratory (8). Human factor Xa, human antithrombin, human thrombin, and human F_{1+2} were purified by techniques established in our laboratory (8, 14, 15). Protein concentrations were determined from the absorbance of protein solutions at 280 nm. The molar extinction coefficients for human prothrombin, human factor Xa, human antithrombin, human thrombin, and human F_{1+2} were assumed to be 13.6, 11.6, 6.5, 16.2, and 12.9, respectively (8, 16).

Collection and processing of blood samples. Venipunctures were performed atraumatically with 19- or 21-gauge butterfly infusion sets using a two-syringe technique. Blood samples were drawn into plastic syringes preloaded with the appropriate solutions as described below. These included: (a) For all functional assays and immunologic measurements of antithrombin, α_1 -PI, and prothrombin, 3.8% (wt/vol) sodium citrate was employed; the ratio of anticoagulant to blood used was 0.1:0.9 (vol/vol). (b) " F_{1+2} anticoagulant": citric acid 7.3 mg/ml, sodium citrate 22 mg/ml, dextrose 24.5 mg/ml, EDTA 2.234 mg/ml, adenosine 1.602 mg/ml, and heparin 25 U/ml. The ratio of anticoagulant to blood employed was 0.2:1.0 (vol/vol). (c) "FPA anticoagulant": This preparation was provided by Mallinckrodt, Inc., St. Louis, MO. The ratio of anticoagulant to blood used was 0.1:0.9 (vol/vol). After collection of blood samples, plasma fractions were obtained by centrifugation at 4°C for 15 min at 1,600 g and stored at -80°C before use.

Normal ranges for antithrombin, α_1 -PI, F_{1+2} , and FPA were derived from data on control subjects. This population consisted of healthy individuals who gave a negative history for bleeding as well as thrombosis, and were not taking any medications at the time of sample collection. A normal plasma pool was constructed by pooling equal volumes of plasma from these control subjects.

Coagulation studies. Routine coagulation studies including prothrombin time and activated partial thromboplastin time were performed by standard laboratory methods (17).

Immunoassays. The plasma concentrations of antithrombin, prothrombin, and F_{1+2} were determined by double antibody RIA as described in earlier reports from our laboratory (9, 18). The plasma levels of FPA were established by RIA utilizing a kit provided by Mallinckrodt, Inc. α_1 -PI levels were measured employing a nephelometric method (19).

Plasma antithrombin functional assays. An amidolytic assay for heparin cofactor activity utilizing the chromogenic substrate Tos-Gly-Pro-Arg-pNA (Chromozym TH, Boehringer Mannheim, Indianapolis, IN) was employed as previously described (20), except that the heparin concentration in the diluent buffer was reduced from 3 to 0.5 U/ml. A ratio of plasma to diluent buffer of 1:59 (vol/vol) was defined as 100% activity. Normal plasma dilutions and samples were each run in duplicate. The activity of test samples was obtained by comparison to a standard curve constructed from dilutions of a normal plasma pool and their respective absorbance readings ($r = -0.99$). The interassay standard deviation for a given plasma sample was 2.9% (30 determinations).

Plasma antifactor Xa functional assays. Plasma antifactor Xa activity was measured in the presence and absence of heparin by employing amidolytic methods similar to those described by Odegard et al. (21). Because human factor Xa and the chromogenic substrate MeO-CO-D-cyclohexyl-glycyl-Gly-Arg-pNA.AcOH (Spectrozyme FXa, American Diagnostica, Greenwich, CT) were utilized in our assay system, it proved necessary to make several modifications in the above protocols as outlined below.

Assays of plasma antifactor Xa activity in the presence of heparin were performed by preparing 1/30 to 1/80 dilutions of normal plasma in buffer (0.05 M Tris-HCl, 0.18 M NaCl, 0.0075 M Na_2EDTA , pH 8.4, containing 3 U/ml heparin). A ratio of plasma to diluent buffer of 1:49 (vol/vol) was defined as 100% activity. Test samples were diluted appropriately with buffer, as judged by their probable antifactor Xa activity. Exactly 200 μl of standard or sample was then transferred into each of two test tubes and prewarmed in a water bath at 37°C

for 2 min. A volume of 100 μl of factor Xa (6 $\mu\text{g}/\text{ml}$ in buffer) was then admixed, and a stopwatch was started. After 10 min of incubation, 50 microliters of substrate (1 mM MeO-CO-D-cyclohexyl-glycyl-Gly-Arg-pNA.AcOH, 0.3 mg/ml polybrene) was added to each tube. Exactly 10 min later, 200 μl of glacial acetic acid was added and the contents were thoroughly mixed. Normal plasma dilutions and samples were each run in duplicate. Absorbances were read at 405 nm against a blank containing 200 μl of the corresponding standard plasma dilution or sample, 150 μl of buffer, and 200 μl of glacial acetic acid. The antifactor Xa activity of test samples was obtained by comparison to a standard curve constructed from the known normal plasma dilutions and their respective absorbance readings ($r = -0.98$). The interassay standard deviation for a given plasma sample was 4.8% (five determinations).

Assays of plasma antifactor Xa activity in the absence of heparin were performed by preparing 1/5 to 1/20 dilutions of normal plasma in buffer (0.05 M Tris-HCl, 0.17 M NaCl, 0.0073 M Na_2EDTA , pH 8.4 containing polybrene 0.05 mg/ml). A ratio of plasma to diluent buffer of 1:9 (vol/vol) was defined as 100% activity. Test samples were diluted appropriately with buffer, as judged by their probable antifactor Xa activity. Factor Xa was diluted to a concentration of 4 $\mu\text{g}/\text{ml}$ in buffer. The assay was then carried out in a manner entirely analogous to that described for the assay in the presence of heparin except that the final incubation lasted only 8 min. The activity of test samples was obtained by comparison to a standard curve constructed from the known plasma dilutions and their respective absorbance readings ($r = -0.97$). The interassay standard deviation for a given plasma sample was 1.5% (five determinations).

F_{1+2} preparation for turnover studies. Human F_{1+2} was purified from 2 liters of fresh frozen plasma obtained from four healthy donors who had been carefully screened for hepatitis and acquired immune deficiency syndrome. The plasma was allowed to warm to 24°C and clotting was initiated by the addition of CaCl_2 (final concentration 32.4 mM). After 90 min of incubation, the clot was removed, heparin (final concentration 1 U/ml) was added, and the suspension was stirred for an additional 5 min. Subsequently, F_{1+2} was isolated from the sera by DEAE-cellulose batch adsorption-elution and hydroxylapatite chromatography as described by Aronson et al. (22). Examination of a series of fractions eluted from hydroxylapatite with a potassium phosphate concentration of 0.35–0.45 M, revealed that >90% of the resultant material was F_{1+2} as judged by the sodium dodecyl sulfate (SDS) gel electrophoretic technique of Laemmli (23). In order to remove minor higher and lower molecular weight components which migrated at rates similar to those of prothrombin and F_1 respectively, 2 mg of F_{1+2} in 15 ml of 0.02 M Tris-HCl, 0.05 M NaCl, pH 7.5, was filtered at 20 ml/h through a column of heparin-Sepharose (0.8×13 cm) equilibrated with the same buffer. The heparin-Sepharose was sterilized during its preparation, and had not previously been used for any other purpose. The matrix was then washed with the above buffer. This procedure was conducted at 4°C. The recovery of F_{1+2} by absorbance measurements averaged 90% of the total amount applied to the column. Analysis of the resultant product by SDS gel electrophoresis confirmed that the minor contaminating species had been eliminated. The overall yield from 2 liters of plasma averaged ~3.6 mg. All buffers were made in sterile, pyrogen-free water, and all glassware had been autoclaved prior to use.

^{131}I - F_{1+2} preparation and turnover studies. Radiolabeling of antigen was accomplished by the method of Greenwood et al. (24) utilizing 15 μg of F_{1+2} and 0.6 mCi of carrier free ^{131}I (New England Nuclear, Boston, MA). Upon completion of the iodination, human serum albumin (Cutter Biological, Berkeley, CA) was added to the reaction mixture (final concentration 10 mg/ml). Separation of free iodide from labeled protein was achieved by filtration of samples at 15 ml/h through a sterile column of Sephadex G-25 (0.8×26 cm) equilibrated with 0.1 M potassium phosphate, pH 7.0. Fractions of 0.5 ml were collected in sterile plastic tubes with screw caps and those within the first peak of radioactivity were examined with respect to their ability to be precipitated with 10% (wt/vol) trichloroacetic acid and specific

F₁₊₂ antisera. Fractions were pooled in which the labeled material was >95% precipitable with trichloroacetic acid and >80% immunoprecipitable by specific F₁₊₂ antisera. This material was frozen at -80°C and utilized within four days of preparation. Prior to intravenous injection, the iodinated F₁₊₂ preparation was brought to a final volume of 3-5 ml in sterile 0.9% (wt/vol) NaCl containing human serum albumin (final concentration 2 mg/ml) and passed through a 0.22-μm filter (Millipore Corporation, Bedford, MA). The labeled material was demonstrated to be sterile by standard microbiologic techniques, and pyrogen-free by the Limulus test (25).

Each subject was given Lugol's solution (three drops twice daily) on the day before injection of ¹³¹I-F₁₊₂ and for the next 10 d in order to prevent uptake of the radioactive iodine by the thyroid. Approximately 40 μCi of ¹³¹I-F₁₊₂ was infused as a bolus into a peripheral arm vein of each volunteer. A 19- or 21-gauge butterfly needle was inserted into a vein on the opposite arm and serial samples of 5 ml of blood were drawn by two-syringe technique into F₁₊₂ anticoagulant. During intervals between specimen collection, a solution of 5% (wt/vol) dextrose was administered to the subjects through this line at a rate of ~50 ml/h. The blood samples were processed and 0.5-ml aliquots of plasma were counted in a Beckman Gamma 8000 Counting System (Beckman Instruments, Inc., Irvine, CA). Exactly 0.25 ml of 20% (wt/vol) trichloroacetic acid was then added to each of the plasmas. The tubes were centrifuged at 1,600 g for 10 min, and the ¹³¹I content of the resultant precipitates was quantitated. Pooled urine samples were collected for each 24-h period throughout the duration of the study. Whole body radioactivity measurements were made by the Massachusetts Institute of Technology Radiation Protection Office utilizing a chair-type shadow shield detector system (26).

Antithrombin concentrates. Antithrombin purified from pooled human plasma of normal donors was supplied by Cutter Biological. After the final purification step, the protein preparation was shown to be homogeneous by SDS gel electrophoresis and migrated as a single band on crossed immunoelectrophoresis in the presence of heparin (27). Each unit of plasma employed in the manufacturing process was nonreactive for hepatitis B surface antigen. In addition, the purified antithrombin was heat-treated to inactivate hepatitis B virus (28).

Informed consent. All clinical studies and informed consent procedures were approved by the Committee on Clinical Investigations, New Procedures and New Forms of Therapy of the Beth Israel Hospital. The procedures utilized in carrying out the ¹³¹I-F₁₊₂ turnover studies were also accepted by the institution's Radioactive Drug Research Committee.

Analysis of data. Estimation of relative immunoreactivity, computation of the slopes of the dose-response curves, as well as determinations of the various associated indices were obtained by a least-squares fit of the RIA results to a "four-parameter" model as described by Rodbard (29, 30). Statistical analyses of data were conducted by standard techniques (31). In most instances, the means are provided with associated standard deviations.

Results

The hemostatic system activity in the blood of patients with congenital antithrombin deficiency was examined. Eight kindreds were available for study where at least two people in each family had previously experienced thrombotic events. The clinical histories and laboratory evaluation of several of these families (Table I; families II, III, and VIII) have been reported previously in the literature (18, 32, 33). Seven of the pedigrees exhibited the "classical" antithrombin deficiency state which is caused by a reduced synthesis of biologically normal inhibitor molecules (34). In these cases, both the immunologic and heparin cofactor activity of antithrombin were reduced to the same extent. Affected members of family VIII possessed the other major subtype of inherited antithrombin deficiency. This disorder is produced by a discrete molecular

Table I. Patients with Congenital Antithrombin Deficiency

Family	Patient	Age, sex	History of thrombosis	AT antigen	AT activity	F ₁₊₂	FPA
				% of normal	% of normal	nM	nM
I	1	22, M	+	36	34	4.01	0.928
	2	14, F	-	41	43	4.20	1.48
II	1	15, M	-	45	37	3.16	2.29
	1	20, F	-	37	37	2.70	0.582
III	1	20, F	-	37	37	3.55	1.14
	1	44, F	-	30	41	2.40	0.625
IV	1	44, F	-	30	41	7.51	1.21
	2	22, M	+	42	31	5.22	1.55
V	3	20, M	+	38	28	3.50	0.996
	4	19, M	-	34	33	3.54	1.76
VI	5	17, M	-	38	36	3.07	1.60
	1	67, F	+	41	48	4.05	1.05
VII	2	39, M	-	46	44	3.26	1.24
	3	38, M	-	45	37	4.35	1.08
VIII	4	34, F	-	59	58	3.63	1.58
	5	30, M	-	40	49	4.67	1.25
IX	6	15, M	-	45	47	4.52	0.995
	7	14, F	-	45	48	5.25	0.671
X	8	12, F	-	41	40	5.06	1.12
	9	4, M	+	49	54	5.22	0.916
XI	1	20, F	-	37	53	3.91	1.29
	1	36, M	-	47	57	4.59	1.08
XII	2	31, F	+	45	46	4.00	1.17
	1	41, F	+	88	43	5.78	3.04
Normal mean						1.97	0.969
±SD						0.97	0.51

Abbreviation: AT, antithrombin.

defect within the protease inhibitor. For this reason, the plasma level of antithrombin in patient VIII-1 as judged by biologic activity measurement is greatly reduced, whereas the immunologic assay of this inhibitor is within the normal range.

A total of 22 antithrombin deficient subjects derived from these kindreds were studied in detail (Table I; Fig. 1). The median age of this cohort on the date of the initial evaluation was 22 yr. All of these individuals were asymptomatic and none were receiving heparin or coumarin derivatives. Only seven of these patients had a prior history of an overt thrombotic episode. Routine coagulation studies including prothrombin time, activated partial thromboplastin time, and platelet count were within normal limits in this population. For comparison, we also provide data on 12 siblings of our antithrombin deficient patients who possessed normal antithrombin levels by immunologic and functional assay (Fig. 1). The median age of this group was 20 yr with a range of 6-55. Plasma

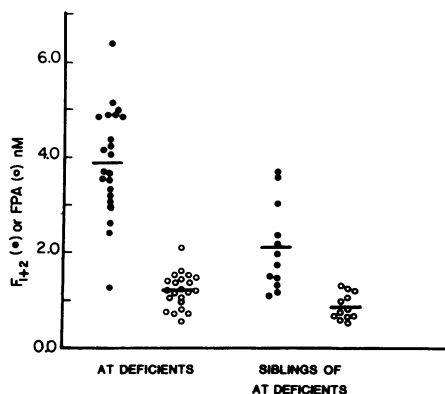


Figure 1. Plasma F_{1+2} (●) and FPA (○) levels in patients with congenital antithrombin deficiency and their unaffected siblings. Horizontal bars represent the mean value of the parameter in each patient group. The mean F_{1+2} and FPA levels in the antithrombin-deficient subjects ($n = 22$) and their unaffected siblings ($n = 12$) were $3.91 \text{ nM} \pm 1.13$ and $1.20 \text{ nM} \pm 0.36$ and $2.07 \text{ nM} \pm 0.92$ and $0.837 \text{ nM} \pm 0.27$, respectively. When concentrations of F_{1+2} and FPA were determined in antithrombin-deficient patients at different time points, the value represents the mean of the two determinations.

prothrombin measurements as determined by RIA in the two groups of subjects were not significantly different from normal (data not shown). The mean level of F_{1+2} in the antithrombin deficient patients was significantly elevated as compared to their unaffected family members or normal individuals, $3.91 \text{ nM} \pm 1.13$ vs. $2.07 \text{ nM} \pm 0.92$ or $1.97 \text{ nM} \pm 0.97$, respectively ($P < 0.001$). It should also be noted that 20 of the antithrombin deficient individuals in this cohort had plasma F_{1+2} values that were $>2.5 \text{ nM}$, while only three of their unaffected siblings (ages 28, 42, and 55 yr) had measurements above this level. The mean plasma FPA concentration of the 22 patients was also significantly increased as compared to their unaffected siblings or normal controls, $1.20 \text{ nM} \pm 0.36$ vs. $0.837 \text{ nM} \pm 0.27$ ($P < 0.005$) or 0.969 ± 0.51 ($P < 0.05$), respectively. However, if one performs this last analysis excluding the single antithrombin-deficient individual with an FPA level $> 2 \text{ nM}$, there is no longer a statistically significant difference between the FPA levels in this group and our normal population.

The levels of F_{1+2} and FPA within the antithrombin-deficient population were not significantly higher among those who had previously sustained thrombotic episodes as compared to their clinically unaffected relatives. Plasma antithrombin activity measurements in the antithrombin deficient patients were tightly clustered from 28% to 58% of that found in the normal population. Linear regression analysis of the data for F_{1+2} or FPA versus antithrombin activity yielded correlation coefficients of -0.12 or 0.18 , respectively. Thus it was not possible to make any meaningful correlations between this latter parameter and the levels of either F_{1+2} or FPA.

The results outlined above suggest that significant elevations in the levels of F_{1+2} occur regularly in patients with clinically asymptomatic antithrombin deficiency, while substantial increases in the concentration of FPA are uncommon within this population. In order to demonstrate that the level of F_{1+2} represents a stable parameter that is characteristic of a particular individual at a given point in time, we have obtained a second blood sample from 10 of the antithrombin deficient patients within 24 mo of the first determination. The second set of

values were similar to those obtained at the initial sampling (Table I). However, we have observed that two of these individuals exhibit a greater amount of variation in their F_{1+2} levels when monitored over a more prolonged period of time (data not shown).

In order to demonstrate that high values of F_{1+2} within the antithrombin deficient population are due to excessive production rather than reduced clearance of this component, the metabolic behavior of this marker was determined in patients with this disorder and compared to that observed in normal individuals. To this end, F_{1+2} was purified from human plasma and radiolabeled with ^{131}I (see Methods). The ^{131}I - F_{1+2} preparations were first shown to be homogeneous by SDS gel electrophoresis. However, it is also critical that the labeled F_{1+2} be prepared in such a manner that the iodination procedure does not significantly alter the in vivo behavior of the radioactive species. We therefore conducted turnover studies of ^{131}I - F_{1+2} in two dogs employing protein preparations and iodination procedures which were identical to those subsequently utilized in the human investigations. The radiolabeled F_{1+2} was infused as a bolus into a peripheral leg vein of a dog, and blood samples were drawn through a catheter placed in the jugular vein into preloaded syringes containing F_{1+2} anticoagulant. Within 5 d of this initial experiment, a second study was carried out employing $250 \mu\text{g}$ of unlabeled protein to raise the plasma level to $\sim 7 \text{ nM}$ in each dog. Samples were obtained at various time points and assayed for F_{1+2} . We had previously noted that dog plasma gives no immunoreactive signal in the RIA. This implies that our F_{1+2} antibody population has essentially no ability to recognize either canine F_{1+2} or canine prothrombin. The plasma radioactivity and immunoreactivity data were plotted against time (Fig. 2); ^{131}I - F_{1+2} plasma radioactivity and F_{1+2} plasma immunoreactivity can each be described by a two-exponential curve, $C_1 e^{-r_1 t} + C_2 e^{-r_2 t}$ (35). The fractional breakdown rate, k_B , was calculated from $[C_1/r_1 + C_2/r_2]^{-1}$ (36). The values for C_1 , r_1 , C_2 , r_2 , k_B in the radioactive and immunologic studies were 0.781, 0.265, 0.214, 9.75, 0.337, and 0.852, 0.334, 0.161, 9.21, 0.389, respectively. Similar results were obtained in a second animal. These investigations revealed that iodination did not affect the metabolic behavior of the native F_{1+2} .

Clearance studies with ^{131}I - F_{1+2} were then undertaken in antithrombin deficient subjects and normal individuals. The results of turnover studies carried out in patients I-1 and V-2 as well as two normal male controls are shown in Fig. 3. The antithrombin-deficient patients selected for study each had plasma measurements of F_{1+2} that were $>4 \text{ nM}$, whereas the normal individuals both had levels of this fragment that were under 2 nM . No substantial differences were noted in the plasma radioactivity measurements of the four individuals. This impression was confirmed by fitting the data obtained during the first 6 h to a two-exponential curve which allowed us to compute several metabolic parameters (Table II). In addition, the number of non-trichloroacetic acid-precipitable counts in the plasma never exceeded 10% of the total amount present during this period. Whole body radioactivity measurements revealed similar patterns in the four patients, and the mean number of residual counts remaining in the subjects at 30 h after the initiation of these studies was 23% of the total infused dose. The amount of radioactivity excreted in the urine during the first 24 h averaged 63% of the total ^{131}I - F_{1+2} administered.

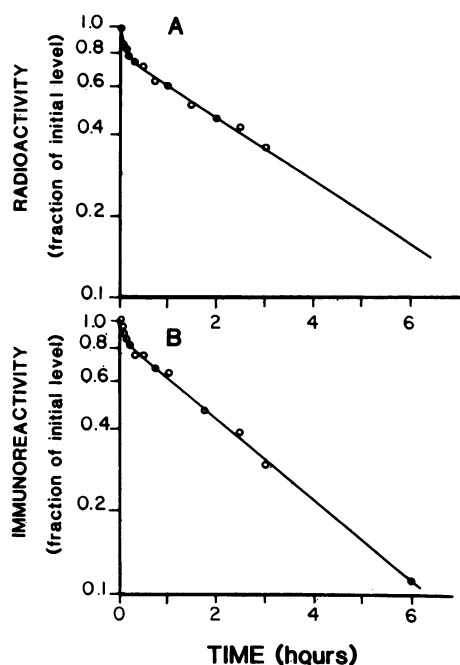


Figure 2. Plasma disappearance of $^{131}\text{I-F}_{1+2}$ and F_{1+2} immunoreactivity in a dog. The animal was infused with $63 \mu\text{Ci}$ of freshly labeled $^{131}\text{I-F}_{1+2}$ (A), and 5 d later received $250 \mu\text{g}$ of unlabeled F_{1+2} (B). Blood samples were drawn at various time points after each infusion. Radioactivity and F_{1+2} measurements are plotted as a fraction of the initial plasma level (taken as the result at the 2-min time point).

The results outlined above allow us to conclude that most individuals with congenital antithrombin deficiency have excessive plasma factor Xa activity as measured by the F_{1+2}

Table II. Parameters from Turnover Studies Using $^{131}\text{I-F}_{1+2}$ in Antithrombin-deficient Patients and Normal Controls

	Antithrombin-deficient		Controls	
	I-1	V-2	1	2
Plasma AT antigen, % of normal	43	46	79	99
Plasma F_{1+2} , nM	4.20	4.52	1.26	1.88
Plasma $^{131}\text{I-F}_{1+2}$ parameters*	C_1	0.764	0.784	0.770
	r_1	0.253	0.270	0.293
	C_2	0.250	0.228	0.227
	r_2	3.15	6.83	5.62
Fractional breakdown rate, h^{-1}	k_B	0.323	0.340	0.375
		0.356		

Abbreviation: AT, antithrombin.

* $^{131}\text{I-F}_{1+2}$ plasma radioactivity is described by a two-exponential curve $\text{C}_1 e^{-r_1 t} + \text{C}_2 e^{-r_2 t}$ (35). Fractional breakdown rate, k_B , was calculated from $[\text{C}_1/r_1 + \text{C}_2/r_2]^{-1}$ (36). When time points greater than 6 h are included in the analysis, an improved curve fit is obtained with parameters generated from the sum of three exponentials; excellent agreement between the parameters in the antithrombin-deficient individuals and the normal controls was again observed. However, we have chosen to express our results in terms of the two-exponential model due to the scarcity of late time points.

assay before the development of overt thrombotic disease. We next undertook studies to specifically correct this increased activity of the hemostatic mechanism by administering antithrombin concentrates to raise the inhibitor levels in these subjects to the normal range. To this end, two antithrombin-

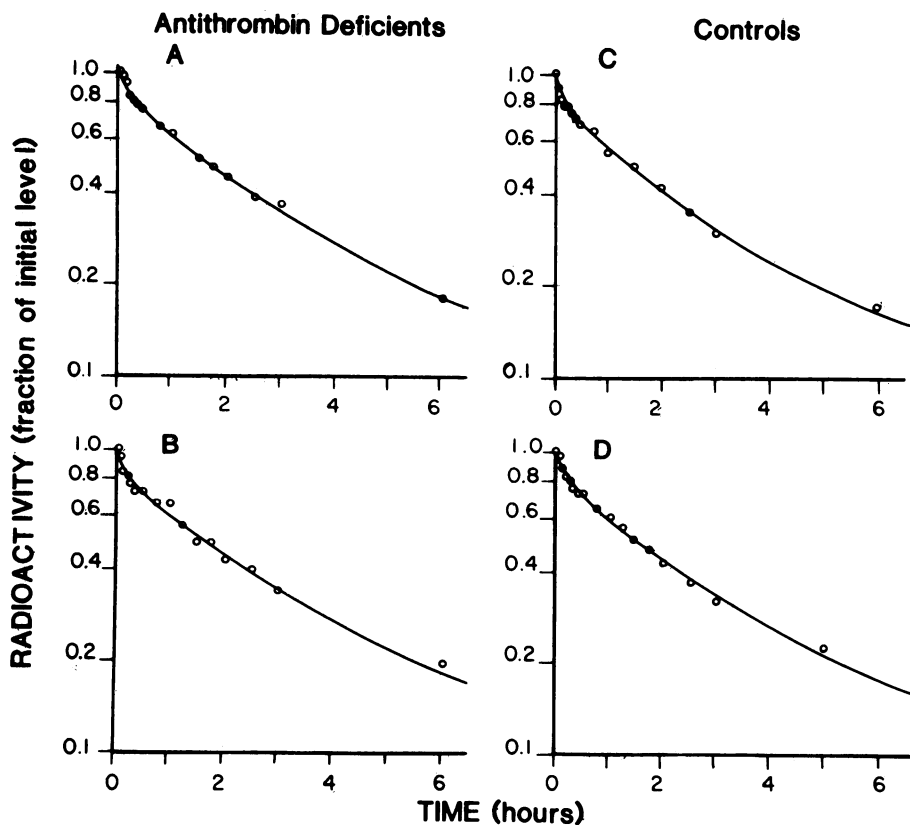


Figure 3. Plasma disappearance of $^{131}\text{I-F}_{1+2}$ in two antithrombin-deficient subjects (A, B) and two normal controls (C, D). Patients A (I-1), B (V-2), C, and D received 41, 29, 42, and $35 \mu\text{Ci}$ of freshly labeled $^{131}\text{I-F}_{1+2}$, respectively, and serial blood samples were taken at various time points after infusion. Radioactivity measurements are plotted as a fraction of the initial plasma level (taken as the result at the 2-min time point).

deficient individuals with elevated levels of F_{1+2} (IV-1, VII-2) were hospitalized in the General Clinical Research Center of the Beth Israel Hospital. Both patients were asymptomatic and neither was receiving anticoagulants at the time of the study. After obtaining base-line blood studies, each subject received an infusion of antithrombin concentrate via a peripheral vein at a dosage of 75 U/kg. One unit is defined as the amount of antithrombin in 1 ml of pooled normal human plasma. Blood samples were obtained by separate venipunctures at various time points from 20 min to 216 h after the infusion was completed. These specimens were assayed for immunologic and functional antithrombin (heparin cofactor activity), F_{1+2} , and FPA (Fig. 4). Our results indicate that the elevated plasma F_{1+2} levels in both patients decreased in response to the infusion of antithrombin. In patient IV-1, the concentration of the activation fragment declined at a rate which was consistent with that predicted from our metabolic clearance studies. Similar profiles demonstrating intermediate values of F_{1+2} between the high preinfusion and nadir postinfusion levels have been observed in other antithrombin-deficient patients. We also observed that the concentration of this marker plateaued in the normal range while antithrombin levels steadily

declined from their peak value after infusion, and started to rise towards the preinfusion value only when the plasma concentration of the inhibitor dropped below $\sim 70\%$ of normal. No significant change in the level of FPA was noted in either individual throughout the duration of the study. Finally, the infused antithrombin was found to have a half-life within the circulation of ~ 48 h which is similar to that obtained in metabolic clearance studies of the radiolabeled protein in humans (34, 37).

It is known that α_1 -PI contributes significantly to the in vitro neutralization of factor Xa by human plasma in the absence of heparin. Gitel et al. (38) have calculated that α_1 -PI contributes 35–40% to the total inhibition of factor Xa in normal human plasma, whereas antithrombin and α_2 -macroglobulin contribute 45–55% and 10–15%, respectively. In order to evaluate further the mechanism by which antithrombin functions as an inhibitor of factor Xa in humans, we have also studied five patients who exhibit severe congenital deficiencies of α_1 -PI with antigen levels $< 5\%$ of normal (Table III). Each of these subjects had previously been genetically typed as PiZ. None was receiving anticoagulants at the time of the examination nor did any exhibit clinical or laboratory evidence of renal dysfunction. Routine serum chemistries to assess liver function were normal in four of the five patients. Patient 5 exhibited evidence of hepatic dysfunction and had low serum albumin as well as plasma antithrombin measurements. We have demonstrated that these individuals show virtually identical decreases in plasma antifactor Xa activity in the absence of heparin when compared to six patients chosen from our antithrombin-deficient population, $75.2\% \pm 7.1$ vs. $69.3\% \pm 6.9$, respectively. However, the plasma F_{1+2} levels and antifactor Xa activity measurements in the presence of heparin of the α_1 -PI-deficient subjects lie within the normal range, $1.72 \text{ nM} \pm 0.49$ and $84.8\% \pm 11.6$, respectively. In contrast, the antithrombin-deficient patients had markedly elevated F_{1+2} levels, low antifactor Xa activity measurements in the presence of heparin, and normal levels of α_1 -PI antigen, $4.20 \text{ nM} \pm 0.61$, $49.2\% \pm 6.3$, and $98.7\% \pm 7.3$, respectively. These data suggest that α_1 -PI does not function as a major inhibitor of factor Xa in vivo and that a tonically active heparin-dependent mechanism exists within the vascular system of humans for accelerating the neutralization of this hemostatic enzyme by antithrombin. Our results are also consistent with the clinical observation that patients with low levels of α_1 -PI do not have an increased incidence of thromboembolic events as compared to the normal population.

Discussion

Numerous investigators have postulated that a hypercoagulable state exists in humans for a prolonged period of time before the development of thrombotic episodes (39). This hypothesis is supported to some extent by various clinical trials in which antiplatelet and anticoagulant medications have been shown to provide some protection against the recurrence of arterial and venous thrombotic disease (40–43). However, a clear biochemical definition of the prethrombotic state has to date proved elusive because of the complex nature of the hemostatic mechanism, the lack of reliable techniques for monitoring pertinent changes in blood coagulability, and the almost exclusive focus upon complicated multifactorial vascular disorders.

Based upon recent advances in our knowledge of the

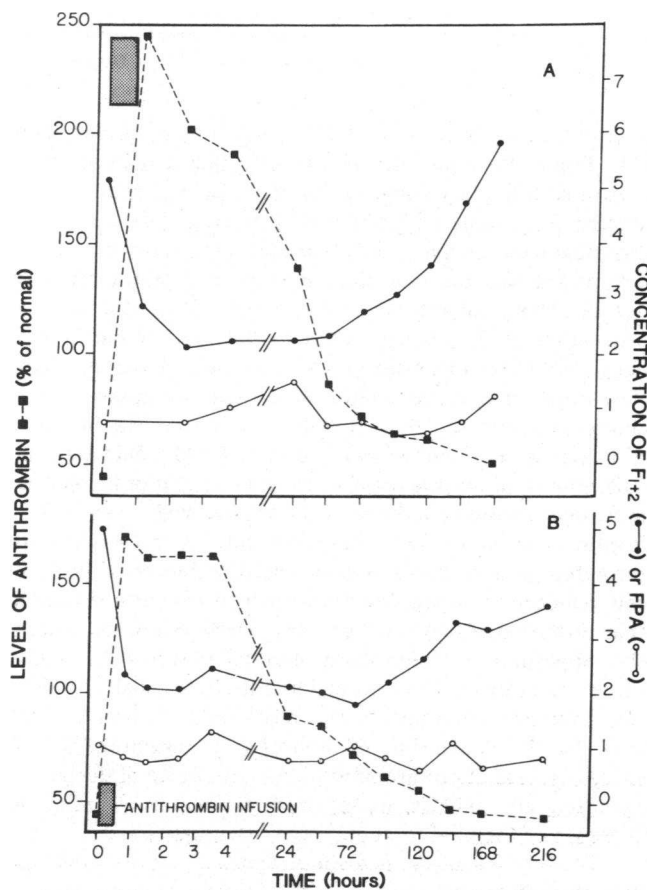


Figure 4. Concentration of antithrombin (■), F_{1+2} (●), and FPA (○) as determined by RIA in patients IV-1 (A) and VII-2 (B) with congenital antithrombin deficiency in response to intravenous infusions of antithrombin concentrates at a dose of 75 U/kg. Patient IV-1 received 7,830 U over 45 min and patient VII-2 received 5,583 U over 20 min. Heparin cofactor assays for antithrombin activity were also performed at all time points, and the measurements obtained were concordant with the levels measured by RIA.

Table III. Comparison of Antifactor Xa Activity and F_{1+2} Levels in Antithrombin and α_1 -Proteinase Inhibitor Deficiency

Patient	Age, sex	AT antigen	Anti-Xa activity with heparin	α_1 -PI antigen	Anti-Xa activity without heparin	F_{1+2}
		% of normal	% of normal	% of normal	% of normal	nM
Antithrombin-deficient						
I-1	22, M	43	51	104	64	4.20
II-1	15, M	45	55	99	83	3.55
IV-4	19, M	42	48	104	70	3.26
V-2	39, M	46	54	107	66	4.52
VII-1	36, M	43	51	87	71	4.85
VIII-1	41, F	88	36	91	62	4.84
Mean			49.2	98.7	69.3	4.20
\pm SD			± 6.3	± 7.3	± 6.9	± 0.61
α_1-Proteinase inhibitor-deficient						
1	42, F	91	90	<5	85	1.43
2	37, M	89	92	<5	77	1.62
3	42, F	87	93	<5	75	1.25
4	36, F	109	87	<5	76	2.65
5	12, M	64	62	<5	63	1.67
Mean			84.8	<5	75.2	1.72
\pm SD			± 11.6		± 7.1	± 0.49

Abbreviation: AT, antithrombin; PI, proteinase inhibitor.

biochemistry of the coagulation system, a series of highly specific immunoassays have been designed that monitor the in vivo activities of various steps of the hemostatic mechanism. In this communication, we have utilized the F_{1+2} and FPA RIAs to characterize the extent of hemostatic mechanism activity in the blood of asymptomatic individuals with congenital antithrombin deficiency. This disorder represents a relatively simple, inherited hypercoagulable state, which is consistently linked with venous and occasionally arterial thrombosis. Indeed, it has been estimated that at least 55% of the individuals with congenital reductions in the levels of this protease inhibitor experience one or more thrombotic events (44).

Our studies were carried out in an antithrombin-deficient population that contained a large percentage of young individuals who had not previously sustained any thrombotic events. The data demonstrate that asymptomatic patients with this disorder usually exhibit increased concentrations of F_{1+2} and that the levels of this species are quite stable over a prolonged period of time within the blood of a given individual. We have also shown that the metabolic behavior of F_{1+2} in antithrombin-deficient patients with high concentrations of this marker are similar to that observed in a normal control population. Therefore, these results allow us to conclude that most individuals with congenital reductions of the protease inhibitor have excessive plasma factor Xa activity for several years before the development of overt vascular disease.

The F_{1+2} turnover studies also permit us to assess quantitatively the extent to which physiologic or pathologic activation of the coagulation mechanism contributes to the catabolism of prothrombin in the circulation of humans. In a steady state, the rate of F_{1+2} production, F , must equal the breakdown rate, B . Under these conditions: F (nanomoles/hour) = B (nanomoles/hour) = $k_B \times V \times C$, where V is the plasma volume of the patient, k_B is the fraction of plasma F_{1+2} broken down per

hour in a steady state, and C is the ambient F_{1+2} concentration (34). Using this equation, we calculate that a normal 70-kg individual (plasma volume ~ 2.8 liters, $k_B \sim 0.365$) with an ambient F_{1+2} value of 2 nM would generate 2.04 nmol/h of this fragment. Shapiro and Martinez (45) have previously determined that the rate of catabolism of prothrombin in a normal 70-kg subject is 0.104 μ mol/h. Thus, the rate of production of F_{1+2} is equivalent to only 2% of the normal daily catabolism of prothrombin on a molar basis. It is also clear from the above relationships that an antithrombin-deficient patient of 70-kg (plasma volume ~ 2.8 liters, $k_B \sim 0.365$) with an ambient F_{1+2} level of 4 nM would generate 4.08 nmol/h of this fragment which is twice that of the normal individual considered above. This rate of production of F_{1+2} is equivalent to 4% of the normal daily catabolism of prothrombin on a molar basis given that the rate of synthesis of this zymogen in an antithrombin-deficient patient is similar to that in the normal population (46). These estimates confirm the supposition of Shapiro and Martinez (45) that the metabolism of prothrombin in normal and hypercoagulable states is predominantly controlled by general catabolic pathways, rather than by the conversion of zymogen to thrombin. Hence, measurements of prothrombin level or clearance of the labeled zymogen are unlikely to be of any value in defining the presence of the prethrombotic state in humans.

The infusion of purified concentrates of antithrombin into two individuals with a congenital deficiency of the protease inhibitor resulted in the normalization of the plasma levels of antithrombin and the concordant correction of hemostatic system hyperactivity as judged by the F_{1+2} assay. It is of particular interest to note that the elevated concentration of the activation fragment in patient IV-1 decreased at a rate which is consistent with that predicted from our metabolic studies of this marker once the plasma concentration of the

protease inhibitor was raised to or above her normal value. Thus, it is highly unlikely that the increased levels of F_{1+2} observed in this hypercoagulable population are produced during venipuncture because of the low ambient concentrations of antithrombin. Indeed, these data directly support the concept that factor Xa and perhaps thrombin are continuously generated within the vascular system of humans.

Marcum et al. (47–50) have provided biochemical, cell biologic as well as physiologic evidence that heparinlike proteoglycans intimately associated with the vascular endothelium can accelerate hemostatic enzyme–antithrombin complex formation via a process that is identical to that of commercial heparin. To provide evidence that this endogenous heparin–antithrombin mechanism is of critical importance with respect to factor Xa inhibition within the circulatory tree of humans, we have compared the results obtained in six antithrombin-deficient individuals with those derived from five patients who exhibited severe congenital deficiencies of α_1 -PI. The plasmas of these two groups of patients show great differences of in vitro antifactor Xa activity in the presence of added heparin but virtually identical decreases of in vitro antifactor Xa activity in the absence of the mucopolysaccharide. The F_{1+2} levels, a measure of in vivo factor Xa activity, within the antithrombin and α_1 -PI-deficient patients are inversely correlated with the in vitro measurements of antifactor Xa activity in the presence of heparin but not in the absence of the mucopolysaccharide. This suggests that anticoagulant active heparinlike species on the luminal surface of the vessel wall activate antithrombin under in vivo conditions, and thereby permit this plasma protein to act as the principal physiologic inhibitor of factor Xa as well as thrombin.

Carlson and co-workers (51) have noted that metabolic turnover studies with radiolabeled antithrombin are consistent with a model in which 10–20% of the total pool of antithrombin is associated with a noncirculating vascular compartment which probably represents the heparinlike proteoglycans of the vessel wall. We believe that antithrombin bound to the vessel wall in an activated conformation constitutes the protease inhibitor population of major physiologic importance whereas the same protein free within the blood is of minimal functional relevance. Therefore, we speculate that antithrombin-deficient patients have an insufficient pool of activated protease inhibitor to adequately suppress factor Xa generated within the circulation thereby resulting in elevated F_{1+2} values. It is impossible at the present time to determine whether this increased action of factor Xa upon prothrombin observed in antithrombin-deficient patients originates on the surface of platelets (52, 53), endothelial cells (54, 55), or leukocytes (56). We also suggest that infusions of antithrombin into such individuals in order to raise their levels of protease inhibitor to >70% of normal lead to the saturation of the heparinlike proteoglycans of the vascular luminal surface with antithrombin. This model would provide an explanation for the decrease in F_{1+2} values which occurs when the concentration of antithrombin is raised to 70% of normal and the subsequent plateau of this parameter despite ambient levels of protease inhibitor that ranged between 70% and 250% of normal.

The antithrombin-deficient individuals also exhibit a mean plasma FPA concentration which is somewhat elevated when compared to that of their unaffected siblings or the normal control population. However, the absolute difference is sufficiently small so that a given assay result in an individual

patient cannot reliably predict the presence of the underlying prethrombotic diathesis. Furthermore, it should also be noted that no change in the levels of FPA were observed in either of the individuals who received infusions of protease inhibitor. Thus, we may conclude that most asymptomatic antithrombin-deficient patients will have augmented prothrombin activation with only a minimal concomitant increase in the apparent activity of thrombin upon fibrinogen.

Several factors must be considered in analyzing this apparently paradoxical situation. The survival of F_{1+2} in the human circulation is quite prolonged as compared to FPA. Based upon our data, we estimate that the clearance rate of F_{1+2} from the plasma is consistent with a $t_{1/2}$ of ~90 min, whereas Nossel et al. (11) have obtained a $t_{1/2}$ for FPA of 3–5 min. However, it is unlikely that this difference in marker catabolism provides the entire explanation for the disparate increase in the levels of F_{1+2} as compared to FPA. Our previous studies of patients with overt thrombotic disease suggest that the F_{1+2} assay is 1.6 times more sensitive than the FPA measurement (57). The antithrombin-deficient patients when compared to the normal population exhibited an average increment in the levels of F_{1+2} of 1.94 nM. This augmentation in prothrombin to thrombin conversion could then be expected to result in a similar average increment in the concentrations of FPA of 1.21 nM. Given that the observed difference in FPA values between the antithrombin-deficient patients and the control population is not >0.23 nM, we must conclude that the action of thrombin upon fibrinogen is markedly suppressed within the vascular system of affected individuals.

Based upon the above considerations, it would appear that antithrombin-deficient patients exhibit a selective defect in the capacity of the endogenous heparin–antithrombin mechanism to inhibit the enzymatic activity of factor Xa, but not thrombin. We are unable at the present time to precisely define the underlying molecular basis for this paradoxical situation. However, two potential explanations can be offered. On the one hand, Jordan et al. (58) have demonstrated with purified components that the rate constant for the neutralization of factor Xa by the heparin–antithrombin complex is 8–10-fold less than that for the inhibition of thrombin by the mucopolysaccharide–protease inhibitor interaction product. If the two hemostatic enzymes were neutralized at similarly disparate rates by antithrombin bound to heparinlike proteoglycans of the vessel wall, then a diminished pool of activated protease inhibitor could result in a significant increase in the concentrations of plasma factor Xa activity without a comparable augmentation in that of plasma thrombin activity. On the other hand, Esmon and co-workers (59–61) have obtained experimental data that indicate that thrombin, but not factor Xa, produced within the vascular system can bind rapidly to the endothelial cell receptor, thrombomodulin. The latter interaction leads to a greatly diminished ability of the bound thrombin to clot fibrinogen as well as an augmented capacity of the enzyme to activate protein C (62). Thus, this second natural anticoagulant mechanism could be responsible for a selective neutralization of thrombin *vis à vis* factor Xa and hence could partially correct the ineffectual inhibition of thrombin but not factor Xa within antithrombin-deficient patients.

It is also important to note that reduced concentrations of antithrombin and elevated levels of F_{1+2} are not always causally linked in a given individual with antithrombin deficiency. This

point is best illustrated by patients III-1 and VI-1, both of whom had F_{1+2} levels < 2.5 nM. Several groups of investigators have demonstrated that activated protein C functions as an inhibitor of blood coagulation by specifically cleaving and destroying factors Va (63–66) and VIIIa (63, 65, 67). It has also been shown that protein S is able to enhance the binding of activated protein C to phospholipid-containing membranes and thereby accelerate the cleavage of the above activated cofactors (68, 69). Therefore, activated protein C in concert with protein S is able to inhibit the factor Xa-factor Va-cell surface dependent conversion of prothrombin to thrombin as well as the concomitant liberation of F_{1+2} (70). We have recently observed that F_{1+2} levels are significantly elevated in nonanticoagulated asymptomatic patients from kindreds with congenital deficiencies of protein C (71) and protein S. These disorders are also highly correlated with an excessive incidence of thromboembolic disease and have clinical presentations similar to that of antithrombin deficiency (72–75). Thus, it seems possible that an excessively active protein C–thrombomodulin mechanism within antithrombin-deficient patients could lead to a normal level of F_{1+2} and the absence of a thrombotic tendency.

In this regard, it is critical to emphasize that most asymptomatic patients with antithrombin deficiency exhibit hemostatic system hyperactivity for prolonged periods of time as quantitated by the F_{1+2} assay and yet the documented occurrence of thrombotic complications within this population is episodic. A compilation of such vascular events in patients with congenital reductions of the protease inhibitor reveals that the first attack occurred in 58% of these patients in conjunction with a process known to trigger thrombosis, such as surgery, pregnancy, delivery, trauma, etc., and was spontaneous in only 42% of this population (44). The biochemical processes which are responsible for the conversion of this hypercoagulable state into an actively thrombotic one are poorly understood at the present time. One might surmise that a variety of events could acutely depress the effective functioning of the endogenous heparin–antithrombin or protein C–thrombomodulin mechanisms. These would include minor alterations in the plasma concentrations of antithrombin or protein C, transient reductions in the levels of heparinlike proteoglycans or thrombomodulin on the surface of endothelial cells, excessive liberation of the platelet inhibitors of these natural anticoagulant mechanisms such as platelet factor four (76), heparitinase (77), or protein C antiactivator (78). The acute dysfunction of the fibrinolytic system may also play an important role in thrombus formation (79).

Despite our uncertainties about various aspects of the pathophysiology of antithrombin deficiency, it is clear that most patients with congenital reductions of the protease inhibitor constitute a population with a true prethrombotic state, i.e., they have excessive production of factor Xa enzymatic activity with insufficient generation of thrombin to convert significant amounts of fibrinogen to fibrin. It remains to be determined whether acute elevations in the concentrations of F_{1+2} will have predictive value in determining the subsequent development of a thrombotic event in antithrombin-deficient subjects. However, because increased levels of this marker are normalized by infusions of antithrombin concentrates in this population, we should be able to utilize our RIA in designing optimal therapeutic regimens for suppressing the hypercoagulable state in individuals with congenital deficiencies of the

protease inhibitor. Finally, the ability of the F_{1+2} assay to define increased activity of the hemostatic system in a population with a simple inherited hypercoagulable disorder suggests that this immunochemical tool can be employed to improve our understanding of other more complex hypercoagulable states as well as to develop effective treatment strategies in these situations.

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