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Effect of Human Granulocytic Elastase on Isolated Human Antithrombin III

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(Received 13 October 1980)

Summary: The interaction of elastase isolated from human granulocytes with purified human antithrombin III was investigated. Antithrombin III did not display any inhibitory effect on granulocytic elastase. Dependent on enzyme concentration, however, granulocytic elastase induced progressive inactivation of antithrombin III leading to an almost complete loss of the thrombin inhibitory activity at a molar ratio of elastase: antithrombin III = 0.4 : 1 within 5 min at 25 °C.

Antithrombin III is not drasticly degraded by elastase as revealed by polyacrylamide gel electrophoretic and rocket immunoelectrophoretic investigations. However, characterization by two-dimensional immunoelectrophoresis with heparin in the first dimensional gel layer revealed a distinct change in the electrophoretic mobility of the inhibitor preincubated with elastase compared to native antithrombin III. This indicates that heparin binding sites of antithrombin III are occupied or affected by the elastase-induced peptide bond cleavage(s). Granulocytic proteinase inhibitors (eglin, Bowman-Birk inhibitor) proved to be highly effective in preventing the antithrombin III inactivation by degradation due to elastase. It is assumed that at least part of the antithrombin III consumption in diseases such as septicemia or endotoxemia is due to proteolysis by granulocytic proteinases. Application of specific inhibitors in the early phase of these ailments should be able to prevent this unspecific degradation of the endogenous antithrombin III.

Wirkung von Elastase aus menschlichen Granulozyten auf isoliertes menschliches Antithrombin III

Zusammenfassung: Es wurde die Interaktion von Elastase, isoliert aus Granulozyten vom Mensch, mit gereinigtem Antithrombin III vom Mensch untersucht. Antithrombin III zeigte keinerlei

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Enzymes:
Chymotrypsin (EC 3.4.21.1);
Cathepsin G = chymotrypsin-like cationic protein from human polymorphonuclear granulocytes (EC 3.4.21.20);
Elastase from human polymorphonuclear granulocytes (EC 3.4.21.11);
Thrombin (EC 3.4.21.5);
Trypsin (EC 3.4.21.4).

Abbreviations:
BzArgNan (L-BAPA) = benzoyl-L-arginine-*p*-nitroanilide;
BBI = Bowman-Birk inhibitor;
IU = inhibitor unit;
Pip = pipecolyl-(piperidine-2-carboxyl-);
Suc[Ala]$_3$Nan (SANA) = 3-carboxypropionyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide.

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Hemmeffekt auf granulozytäre Elastase. Hingegen induzierte granulozytäre Elastase abhängig von der Enzymkonzentration eine zunehmende Inaktivierung von Antithrombin III. Ein nahezu vollkommener Verlust der Thrombinhemmaktivität konnte bei einem Molverhältnis von Elastase zu Antithrombin III von 0.4 : 1 innerhalb von 5 min bei 25 °C Inkubationstemperatur erzielt werden.

Untersuchungen mit Hilfe von Polyacrylamid-Gelelektrophorese und Rocket-Immunelektrophorese zeigten jedoch, daß Antithrombin III durch Elastase nur geringfügig abgebaut wurde. In der zweidimensionalen Immunelektrophorese, mit Heparin im Gel für die elektrophoretische Trennung in der ersten Dimension, wandert der mit Elastase modifizierte Inhibitor hingegen deutlich langsamer als natives Antithrombin III. Dies weist darauf hin, daß Elastase die Heparin-Bindungsstellen des Antithrombin III besetzt oder durch Hydrolyse von Peptidbindungen modifiziert hat. Inhibitoren (Eglin, Bowman-Birk-Inhibitor) granulozytärer Proteinasen verhinderten sehr effektiv den durch Elastase induzierten Abbau und die damit verbundene Inaktivierung von Antithrombin III. Daher kann man annehmen, daß zumindest ein Teil des Antithrombin-III-Verbrauchs bei Septikämien oder Endotoxinämien auf eine Proteolyse durch granulozytäre Proteinasen zurückzuführen ist. Die therapeutische Anwendung spezifischer Inhibitoren in der Frühphase dieser Krankheiten könnte deshalb den unspezifischen Abbau des endogenen Antithrombin III wirkungsvoll verhindern.

Key words: Granulocytic elastase, antithrombin III, proteinase inhibitor, septicemia.

Some years ago, Schmidt et al.[1] demonstrated that human polymorphonuclear leukocytes contain neutral proteinases (elastase-like and chymotrypsin-like) which degrade several clotting factors in vitro. High levels of circulating proteinase-inhibitor complexes in diseases such as leukemia or septicemia[2,3] indicate that direct proteolytic degradation of coagulation factors by liberated leukocytic proteinases may play an important role in consumption coagulopathy throughout these ailments. This assumption is supported by studies showing the release of active neutral proteinases from polymorphonuclear leukocytes in the presence of endotoxin[2,3]. Furthermore, recent results from our laboratory on experimental endotoxemia demonstrate clearly that prior systemic application of an elastase-cathepsin G inhibitor from soybeans (Bowman-Birk inhibitor) prevents the endotoxin-induced degradation of clotting factors and other plasma proteins to a significant degree[4]. This indicates that the functional activity of the endogenous inhibitor potential is considerably overstressed in the case of an increased pathophysiological release of leukocytic proteinases.

It is well known that the risk of thrombosis increases extensively with decreasing plasma levels of antithrombin III, the most important inhibitor of the clotting system[5,6,7]. As the antithrombin III level is very low in septic shock or endotoxemia[4,8], we were interested to find out whether antithrombin III may be consumed by unspecific enzymatic degradation in addition to the regular inactivation of proteinases by formation of proteinase-inhibitor complexes. Therefore, we investigated in some detail the interaction of purified human granulocytic elastase with purified human antithrombin III as well as the presumable protective effect of elastase-cathepsin G inhibitors (eglin, BBI).

Material and Methods

a) Enzyme activity and inhibition assays

Granulocytic elastase

Human granulocytic elastase (M_r ~ 30000) was isolated according to the method of Baugh and Travis[9]. The lyophilized proteinase was stored at + 4 °C. Enzyme solutions were freshly prepared with 0.2M triethanolamine buffer of pH 7.8. Elastase amidolytic activities were measured using the chromogenic substrate Suc-[Ala]$_3$Nan (SANA, Protein Research Foundation, Osaka, Japan, no. 740813). The procedure followed was that described by Schiessler et al.[10]. Protein concentration was determined spectrophotometrically by the microassay procedure of Bio-Rad Laboratories, Richmond, California according to M. Bradford[11]. A specific activity of ~ 0.3 U per mg protein was determined for the purified elastase.

Antithrombin III

Antithrombin III, a research product of Behringwerke AG, was isolated by means of affinity chromatography using insolubilized heparin. A stock solution was made by solving the lyophilized product in dist. water. This solution was divided into small portions and stored at – 20 °C until use. Further dilutions were made with 0.2M triethanolamine, pH 7.8. The inhibitory activity of a 50 *μl* stock solution corresponded to the antithrombin III activity of 1 m*l* normal human plasma when assayed with the amidolytic substrate.

Inhibition of the amidolytic thrombin activity by antithrombin III was measured by the initial rate method at 25 °C using the chromogenic peptide substrate S-2238 (H-D-Phe-Pip-Arg-Nan • 2 HCl) which was a generous gift of Deutsche Kabi, München. For determination, one part of the incubation mixture (25 *μ*g antithrombin III per 100 *μl* 0.2M triethanolamine, pH 7.8) was mixed with nine parts of an appropriate heparin buffer, pH 8.4 (0.5mM Tris, 7.5mM $Na_2EDTA \cdot 2 H_2O$, 175mM NaCl, 3000 units heparin/*l*). Further dilutions were made according to the commercial company's instructions (Table 1). The final antithrombin III-assay mixture (800 *μl*) consisted of 370 *μl* heparin buffer, 30 *μl* diluted antithrombin III sample, 100 *μl* (0.18 nkat) thrombin solution and 300 *μl* (0.75mM) S-2238 solution. The ΔA/min values remained constant during at least 6 min. Antithrombin III activities corresponding to the different dilution steps of each sample were expressed as described by Kabi's instructions and are outlined in Table 1.

Elastase-cathepsin G inhibitors

The elastase-cathepsin G inhibitor from soybeans (type: Bowman-Birk, $M_r \sim 8000$) was isolated from a raw extract "trypsin inhibitor from soybeans" (Serva, Heidelberg. no. 37340) by gel filtration on Sephadex G-75 (Pharmacia, Uppsala, Sweden) with 2% (v/v) acetic acid as eluant. The inhibitor-containing fractions were sampled, freeze-dried and redissolved in 0.2M triethanolamine, pH 7.8. Their specific trypsin inhibitory activity was estimated to 3.0 IU/mg protein. The procedure followed was that described by Fritz et al.[12] with benzoyl-L-arginine-*p*-nitroanilide (L-BAPA, no. 10754) as substrate and bovine trypsin (no. 24581), both from E. Merck, Darmstadt.

Eglin ($M_r \sim 8000$) from leeches was a gift from *Dr. Seemüller* from this laboratory[13]. The lyophilisate, solved in 0.2M triethanolamine, pH 7.8, showed a specific chymotrypsin inhibitory activity of 0.1 IU/mg protein[12] with 3-carboxypropionyl-L-phenylalanine-

Table 1. Conditions used to establish a standard curve of antithrombin III activity in samples containing either normal human plasma or purified human antithrombin III.

One part of plasma or antithrombin III solution (25 *μ*g antithrombin III per 100 *μl* 0.2M triethanolamine, pH 7.8) was mixed with nine parts of heparin buffer, pH 8.4 (0.5mM Tris, 7.5mM Na_2-EDTA • 2 H_2O, 175mM NaCl, 3000 units heparin/*l*). Further dilutions were made by mixing each time 50 *μl* of the prediluted samples with different amounts of heparin buffer (see table, column 2). For antithrombin III activity determination, 30 *μl* of these various inhibitor-containing solutions were incubated with 370 *μl* heparin buffer, 100 *μl* (0.18 nkat) thrombin and 300 *μl* (0.75mM) peptide substrate S-2238 ("final assay mixture", 800 *μl*). For more details, see text.

Dilution step	Heparin buffer	Final assay mixture	
		Plasma samples	Purified antithrombin III samples
	[*μl*]	Equivalent antithrombin III activity[a] [%]	Protein content [ng]
1	1150	25	31.2
2	550	50	62.5
3	350	75	93.7
4	250	100	125.0
5	190	125	156.2

[a] Definition according to the instructions of Kabi Diagnostica (Stockholm, Sweden).

p-nitroanilide (no. 15070) as substrate and bovine chymotrypsin (no. 2308), both from E. Merck.

b) Incubation conditions

Antithrombin III in the proper final dilution of 25 μg protein per 100 μl incubation medium (0.2M triethanolamine, pH 7.8) was incubated with various amounts of elastase in the ratios 0.01, 0.02, 0.04 and 0.4 mol elastase per mol antithrombin III (assuming $M_r \sim 30000$ for granulocytic elastase and $M_r \sim 65000$ for antithrombin III) at 0 °C, 25 °C and 37 °C. The reaction was stopped after different time intervals (5, 15 and 60 min) by immediate deep-freezing of appropriate samples. To investigate the protective effect of eglin or BBI, elastase was preincubated with an excess of inhibitor (2 mol inhibitor per 1 mol elastase) to achieve complete inactivation of the enzyme before mixing it with antithrombin III. The antithrombin III activity was determined as described under "enzyme activity and inhibition assays" after re-thawing the incubation mixtures. The dilution steps performed for each sample are outlined in Table 1. The degree of antithrombin III inactivation was calculated using the inhibitory activity of 156 ng antithrombin III in an elastase-free control sample as reference. This amount of the inhibitor decreased the amidolytic activity of 0.18 nkat thrombin almost completely.

c) Dodecyl sulfate and conventional polyacrylamide gel electrophoresis

Dodecyl sulfate electrophoresis under reducing (with 1% 2-mercaptoethanol) or non-reducing conditions and conventional polyacrylamide gel electrophoresis were performed as horizontal flat gel electrophoresis with the LKB 2117 Multiphor apparatus according to the LKB application note 306. The thickness of the gels was 2 mm. To obtain better resolution, we also used ultrathin-layer gels (0.48 mm) as described by Görg et al.[14]. The applied sample volumes were 10 μl or 2.5 μg antithrombin III protein for the 2 mm gels and 4 μl antithrombin III for the 0.48 mm gels. In each case, the 10% gels were subjected to electrophoresis at 6.5–7.5 V/cm for 5 h (sodium dodecyl sulfate gels) or 10 V/cm for 4 h (conventional polyacrylamide gel electrophoresis). After protein fixation for 1 h with 20% (w/v) trichloroacetic acid staining was performed with Coomassie Brillant Blue R 250 (Serva GmbH, Heidelberg, no. 17525).

d) Immunoelectrophoresis

Rocket immunoelectrophoresis was conducted according to Laurell[15] in 1% agarose on plastic plates from LKB, Sweden (no. 2117–406) using agarose tablets (no. 170–3003) and Tris/tricine* buffer, pH 8.6 (no. 170–3005) from Bio-Rad Laboratories, Richmond, California. Electrophoresis was carried out at 8–10 V/cm for 4 h. The monospecific antithrombin III antiserum was purchased from Behringwerke, Marburg (ØTPV 04 Clotimmun-Antithrombin III) and applied in a final concentration of 1%. Amounts of 5 μl, i.e. ~ 1.25 μg antithrombin III protein, were applied per sample. The gel plates were stained with Coomassie Brillant Blue R 250. Two-dimensional immunoelectrophoresis[16] in 1% agarose was performed on two different sizes of plastic plates from LKB, Sweden (no. 2117–406 with 8.4 x 9.4 cm and no. 2117–407 with 18 x 9.4 cm). The sample application wells were located 1.5 cm (smaller plates) and 8.0 cm (larger plates) from the edge[17]. Heparin (Heparin Vitrum, Deutsche Kabi, München, no. H 1505) in the appropriate concentration of 4 units/ml agarose solution[17] was carefully mixed with the fluid 1% agarose gel before making the first-dimensional gel layer. Electrophoresis in the first dimension was run for 2 h and in the second, antibody-containing gel layer for 4 h using 8–10 V/cm each time. Agarose, electrophoresis buffer, applied sample volumes and antiserum in the second layer were the same as mentioned for rocket immunoelectrophoresis. All further treatments for two-dimensional electrophoresis were the same as described in detail by Ganrot[16].

* N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine

Results

Antithrombin III inactivation as measured with enzymatic assays

In a first approach the thrombin inhibitory properties of purified antithrombin III and of a control plasma from normal individuals were compared. If appropriate concentrations of purified antithrombin III and control plasma were applied to the amidolytic assay, almost identical inhibition curves were obtained (Fig. 1). To achieve proper inhibition of the given amount of thrombin (0.18 nkat) an excess of antithrombin III over the equimolar enzyme-inhibitor ratio is necessary, cf. Fig. 1. For practical reasons, the inhibitory activity value of the sample containing 156 ng antithrombin III, corresponding to a 1.25-fold antithrombin III excess over the equimolar ratio (or "125%" inhibition according to the instructions of Kabi Diagnostica, Stockholm, Sweden, Table 1) was used as reference for the antithrombin III samples incubated with elastase or the elastase-inhibitor mixture.

Antithrombin III did not display any inhibitory effect on granulocytic elastase in the amidolytic

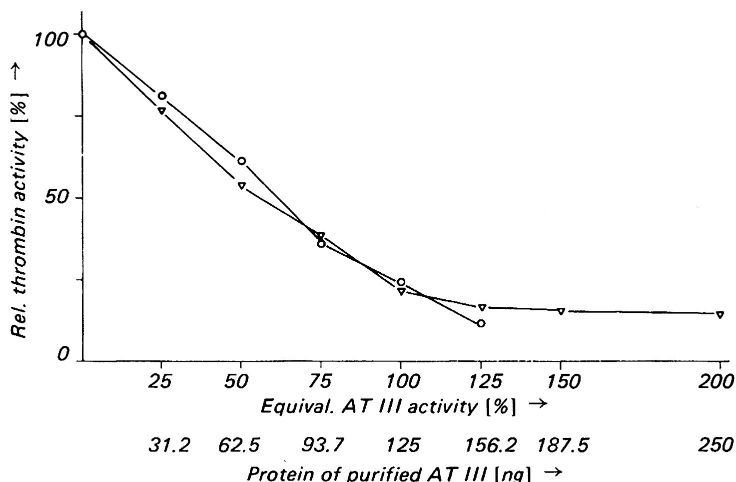

Fig. 1. Standard curve of the thrombin inhibitory property of antithrombin III (AT III) as obtained with appropriate solutions of purified AT III (o——o) or normal human control plasma (▽——▽) from Kabi Diagnostica, Stockholm, Sweden).

For experimental details, see "Material and Methods" and Table 1.

assay (substrate Suc[Ala]$_3$Nan). However, antithrombin III lost its activity almost completely in the presence of elastase at a molar ratio of elastase: antithrombin III = 0.4 : 1 (Table 2). Incubation times even shorter than 5 min (data not shown) were long enough to inactivate more than 95% of the given antithrombin III sample. Furthermore, the degree of inactivation was almost independent of the incubation temperature, indicating a very rapid reaction also at 0 °C. On the basis of these data the incubation conditions for further investigations were settled upon 5 min at 25 °C.

In Fig. 2 (block diagram 1), the degree of antithrombin III inactivation is shown as a function of the applied concentration of granulocytic elastase. Decreasing amounts of elastase resulted in less extensive antithrombin III inactivation, but even catalytic amounts of elastase (4 to 1 mol/100 mol) led to an appreciable loss in antithrombin III activity within 5 min at 25 °C. This inactivation could neither be prevented nor accelerated by addition of heparin to the incubation mixtures.

Table 2. Effect of granulocytic elastase on antithrombin III activity (molar ratio of elastase: antithrombin III = 0.4 : 1) as function of incubation period and incubation temperature. For experimental details, see "Material and Methods".

Incubation		Antithrombin III activity
Period [min]	Temp. [°C]	[%][a]
15	0	5.7
60	0	5.6
5	25	3.8
15	25	5.2
15	37	2.6
60	37	8.6

[a] Related to the elastase-free reference value.

To show whether the antithrombin III-thrombin complex is also attacked by elastase thereby releasing active thrombin, we used the assay conditions outlined in Table 3. The results demonstrate clearly that antithrombin III remained bound to thrombin.

Furtheron, the effect of elastase on the antitryptic activity of antithrombin III[18,19,20] was investigated. This antitryptic activity was determined by measuring the inhibition of the trypsin-catalyzed hydrolysis of BzArgNan as described by Fritz et al.[12]. In this assay system trypsin activity was completely inhibited by short incubation (5 min) of the enzyme (10 μg protein) with antithrombin III (15 μg protein). Preincubation of antithrombin III with granulocytic elastase (in a molar ratio of 0.4 : 1 for 5 min at 25 °C) caused complete loss of the antitryptic activity of antithrombin III.

Prior incubation of elastase with the granulocytic elastase-cathepsin G inhibitors, eglin and Bowman-Birk inhibitor, respectively, abolished the inactivating effect of elastase on antithrombin III activity to various extents (second and third block diagram in Fig. 2).

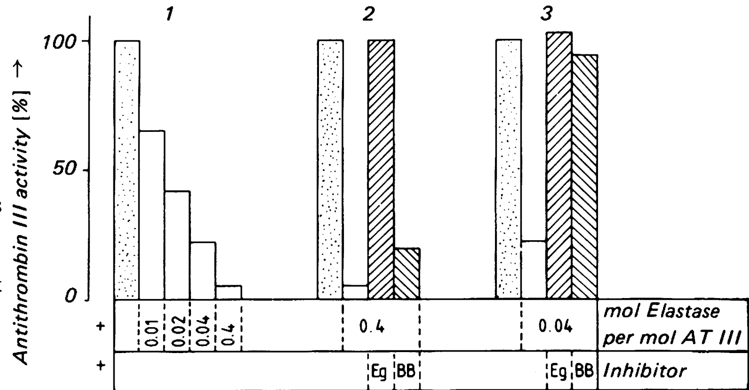

Fig. 2. Inactivation of purified antithrombin III (AT III) by purified granulocytic elastase and protective effect of granulocytic proteinase inhibitors (eglin, Bowman-Birk inhibitor).
Block diagram 1: Gradual loss of AT III activity by increasing amounts of elastase.
Block diagram 2 and 3: Protective effect of eglin (Eg) and Bowman-Birk inhibitor (BB) after prior incubation of elastase with the inhibitors.
For experimental details, see "Material and Methods".

It should be emphasized that in the concentration applied neither elastase nor eglin or BBI interfere with the antithrombin III assay. Whereas eglin proved to be fully effective at each elastase concentration applied, BBI showed a surprisingly weak protection in samples with the highest elastase concentration though a 2-fold molar excess of the inhibitor over elastase was used. This might be explained by the fact that eglin is supposed to be an irreversible inhibitor of granulocytic elastase whereas BBI inhibits the proteinase reversibly. Similar observations were made when the same samples were analyzed by two-dimensional immunoelectrophoresis (Fig. 4d, e).

Antithrombin III inactivation as followed by electrophoretic analyses

Purified antithrombin III migrated as two barely separable bands on conventional gel electrophoresis (Fig. 3, sample 1). The presence of a faint secondary band in our preparation might be due to antithrombin III microheterogeneity or to

Table 3. Effect of granulocytic elastase on antithrombin III activity before and after incubation of antithrombin III with thrombin. All samples contained 156 ng antithrombin III protein in 30 *µl* heparin buffer. Further details see "Material and Methods".

Steps in procedure		Control sample	Samples preincubated	
			without thrombin	with thrombin
Incubation mixture				
heparin buffer	[µl]	370	360	360
antithrombin III	[µl]	30	30	30
thrombin	[µl]	100 (0.18 nkat)	–	100 (0.18 nkat)
elastase	[µl]	–	10 (29 ng protein)	–
Preincubation conditions			5 min, 25 °C	5 min, 25 °C
Enzyme addition				
thrombin	[µl]	–	100 (0.18 nkat)	–
elastase	[µl]	–	–	10 (29 ng protein)
Incubation		10 min, 25 °C	5 min, 25 °C	5 min, 25 °C
S-2238 addition	[µl]	300	300	300
Calculation of antithrombin III activity	[%]	100	1.3	98.2

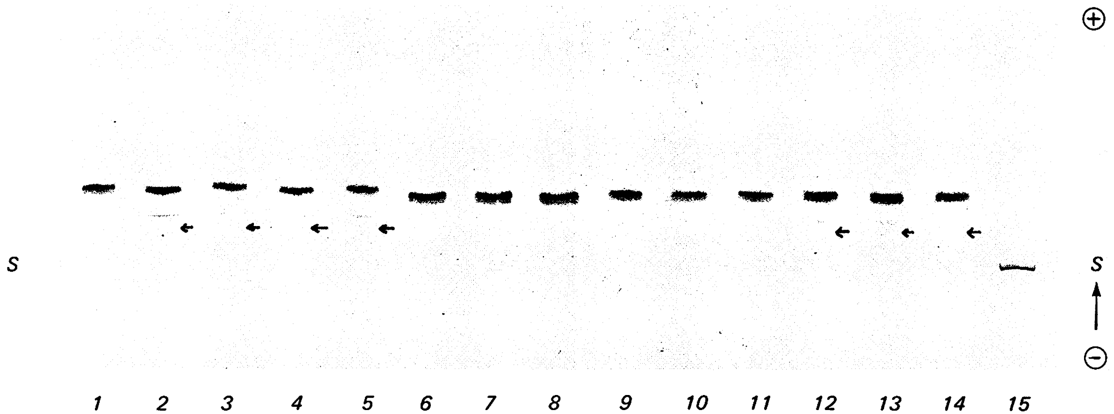

Fig. 3. Conventional polyacrylamide gel electrophoresis on a 10% horizontal flat gel, 0.48 mm thick. Comparison of the electrophoretic behaviour of purified granulocytic elastase (sample 15), purified antithrombin III (AT III) (sample 1), AT III plus granulocytic elastase (samples 2–5 and 12–14), AT III plus granulocytic elastase pretreated with the proteinase inhibitors eglin (samples 6–8) or BBI (samples 9–11).

Incubation of AT III with elastase (molar ratio 1:0.4 or 1:0.04) resulted in the occurrence of two additional protein bands (samples 2–5 and samples 12–14), one of them being so faint (see small arrows) that it could be detected only at the gel but not visualized at the photograph.

Samples were applied at the position marked with 'S'. For further experimental details, see "Material and Methods".

small amounts of inactive antithrombin III-derived products (cf. "Discussion"). Incubation of elastase and antithrombin III in a molar ratio of 0.4:1 (Fig. 3, samples 2–5) or even 0.04:1 (Fig. 3, samples 12–14) resulted in the occurrence of two additional protein bands (one of them very faint and hardly visible) with slower mobility, whereas the major antithrombin III band had moved a little faster and its secondary band had disappeared. Under the conditions used for electrophoresis, elastase itself did not migrate at all (Fig. 3, sample 15) as shown with a concentration which was much higher than that applied in the incubation mixtures with antithrombin III. Moreover, the elastase concentrations in these incubation mixtures were too low to produce any protein bands on the gels. So it seems very unlikely that the additional bands observed in mixture with elastase should be due to degradated elastase. Inhibition of elastase with eglin or BBI prior to incubation with antithrombin III led to a protein pattern (Fig. 3, samples 6–8 and 9–11) identical with that of untreated antithrombin III.

Separation of the various samples by dodecyl sulfate electrophoresis under non-reducing conditions revealed one protein band for untreated antithrombin III and eglin- or BBI-protected antithrombin III, respectively, whereas two barely separable bands arose after elastase-treatment of antithrombin III (results not shown here). A more complex pattern of protein bands (at least three) was obtained if the samples were subjected to dodecyl sulfate electrophoresis under reducing conditions. The interpretation of these protein patterns, however, deserves supplementary and more sophisticated investigations.

To characterize further antithrombin III-related products generated by granulocytic elastase, two-dimensional immunoelectrophoresis was performed. It is well-known that free and complexed forms of antithrombin III differ significantly in their electrophoretic mobility in the presence of heparin so that they may be differentiated by this method[17]. When the purified product was subjected to two-dimensional immunoelectrophoresis with 4 units heparin/m*l* agarose gel in the first dimensional run, two peaks with antithrombin III antigenicity appeared (Fig. 4a). The pattern of precipitin peaks obtained when a mixture of equimolar amounts of antithrombin III and the

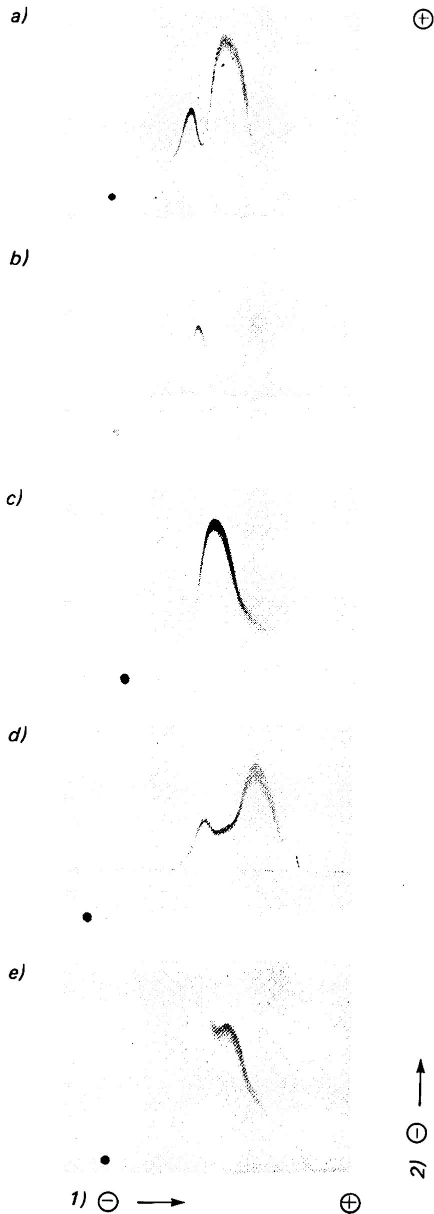

Fig. 4. Two-dimensional immunoelectrophoresis of purified antithrombin III (AT III) a), AT III plus AT III-thrombin complex b), AT III plus granulocytic elastase c), and AT III plus elastase pretreated with eglin d) or BBI e) in 1% agarose containing 4 U heparin/m*l* in the first dimensional layer. The monospecific AT III-antiserum in the second dimensional layer was applied in a final concentration of 1%.

For further experimental details, see "Material and Methods".

antithrombin III-thrombin complex was applied is shown in Fig. 4b. In the latter case the left peak corresponds to the antithrombin III-thrombin complex, whereas the nature of the antithrombin III product corresponding to the minor left peak of Fig. 4a is not yet known. Treatment of antithrombin III with elastase (molar ratio 1 : 0.4) caused complete shift of the major precipitin peak of native antithrombin III towards the position of its original minor peak or that of the antithrombin III-thrombin complex (Fig. 4c). Preincubation of elastase with eglin or BBI before starting the reaction with antithrombin III resulted in a more (eglin, Fig. 4d) or less (BBI, Fig. 4e) complete restoration of the two-peak precipitin pattern of native antithrombin III. This might also be due to a reversible inhibition of granulocytic elastase by BBI compared to the irreversible inhibition by eglin as already discussed.

In rocket immunoelectrophoresis all samples (antithrombin III, antithrombin III plus elastase, antithrombin III plus elastase pretreated with eglin or BBI) caused only one precipitin rocket (data not shown) so that a differentiation by this method is not feasible. In all cases even the heights of the rockets were of the same range, indicating that in contrast to the antithrombin III activity the immunogenicity of antithrombin III was not significantly altered by elastase treatment.

Discussion

The major inhibitor of clotting factors, antithrombin III, is of vital importance for the homeostatic balance of the clotting system. Heriditary or acquired antithrombin III deficiency is normally associated with increased thromboembolic risk. Therefore, consumption and inactivation of antithrombin III by degradation due to proteinases, which have access to the circulation in the course of certain diseases, are of special medical interest. Only recently we suggested that in addition to clotting factors and other plasma proteins, plasma proteinase inhibitors might be also consumed unspecifically in a similar way during septicemia, septic shock or endotoxemia[4,8].

Indeed, as shown in the present report, purified antithrombin III is rapidly inactivated by granulocytic elastase thereby loosing its inhibitory

activity on the amidolytic and probably also the clotting activity[21] of thrombin. Even catalytic amounts of elastase are highly active under given conditions: antithrombin III looses 35 resp. 80% of its activity within 5 min at 25 °C in the presence of 1 resp. 4 mol elastase/100 mol (Fig. 2). The functional integrity of the purified antithrombin III is demonstrated by the coincidence of the inhibition curves obtained with isolated antithrombin III and a control plasma, respectively (Fig. 1).
Inactivation of antithrombin III with 40 mol elastase/100 mol was used to reach a final degradation state within a short time, and to see whether extensive degradation occurs. However, as revealed by the polyacrylamide gel electrophoretic (Fig. 3) and rocket immunoelectrophoretic (data not shown) investigations antithrombin III is not drasticly degraded by elastase. Preliminary results of more sophisticated studies indicated that not more than two peptide bonds are cleaved (M. Jochum, R. Geiger, N. Heimburger, H. Fritz, 1980, unpublished observations). Such a limited degradation would also explain the similarity in the electrophoretic mobility and the immunogenicity of the proteolytically modified and native antithrombin III. On the other hand, the change in the electrophoretic mobility of the modified inhibitor, compared to native antithrombin III in the presence of heparin (Fig. 4a, c), indicates strongly that heparin-binding sites of antithrombin III are affected or even eliminated by the elastase induced peptide bond cleavage(s). Remarkably, the modified inhibitor has a similar mobility in the presence of heparin as the thrombin-antithrombin III complex (Fig. 4b). Actual evidence suggests that qualitative or quantitative differences in heparin binding by free or complexed antithrombin III are chiefly responsible for the different electrophoretic mobilities of native and complexed antithrombin III, respectively[17,22–24]. The reason seems to be the presence of a proteolytically modified inhibitor in the complex, since the free modified (i.e. inactive) inhibitor also shows considerably reduced affinity to heparin[23]. We would like to speculate, therefore, that the peptide bond(s) cleaved by elastase in antithrombin III are in close vicinity or even identical with the bond cleaved by thrombin or trypsin during complex formation. This view is supported by the inability of elastase to attack antithrombin III when it is bound to thrombin in the complex. In this case the reactive site peptide bond of antithrombin III is not accessible to elastase due to steric hindrance. Two further observations of this study may be explained on the given basis: (i) The barely separable minor protein band visible in conventional gel electrophoresis (Fig. 3) should be due to the presence of a proteolytically modified antithrombin III in our purified antithrombin III preparation.
(ii) The occurrence of the two-peak pattern in two-dimensional immunoelectrophoresis (with heparin in the first dimensional gel layer) is consistent with the findings of Barrowcliffe[24], who obtained similar patterns using various samples of purified antithrombin III. He provided clear evidence that a portion of the preparation does not bind to heparin and may represent therefore the slower moving peak of the two-component pattern. There are, however, divergent views[24] as to whether this non-heparin binding fraction is still biologically active.
The nature of the modified antithrombin III protein is evident from more recent investigations. Concurrently with the formation of the thrombin-antithrombin III complex a proteolytically modified, inactive form appears[23]. The latter consists of two polypeptide chains connected by disulfide bridges[22]. This modified antithrombin III could not be differentiated from the equally inactive protein released by dissociation of the thrombin-antithrombin III complex, suggesting that limited proteolysis of antithrombin III by thrombin is involved in complex formation. Similar results were obtained during complex formation of antithrombin III with trypsin[20]. On the other hand, it was recently shown[25] that protein proteinase inhibitors are preferably cleaved at their reactive site peptide bonds by proteinases not inhibited by them. Obviously, the reactive site bond of an inhibitory protein may be highly susceptible to proteolysis, at least in some cases including α_1-antitrypsin[26,27]. This might also apply to the elastase-antithrombin III system. Though antithrombin III does not inhibit granulocytic elastase at all, its thrombin- or trypsin-directed reactive site bond could be cleaved preferentially by this enzyme. However, more detailed studies have to verify this concept.
In diseases like septicemia or endotoxemia extensive consumption of antithrombin III is obser-

ved[4,8]. Although in vitro effects can be transferred to in vivo conditions only with reservation, we assume now that at least part of antithrombin III consumption is due to proteolysis by granulocytic and other proteinases released into the circulation by the effect of endotoxins. This assumption is emphasized by the fact that the loss of antithrombin III throughout experimental endotoxemia was to a large extent prevented under systemic medication with BBI[4]. BBI[10] and especially eglin, the most potent protein inhibitor of human granulocytic elastase and cathepsin G in our hands[13], proved to be highly effective in preventing antithrombin III degradation by elastase also in this study. In our opinion, application of such inhibitors in the early phase of septicemia may not only significantly prevent unspecific degradation of plasma factors such as the enzymes of the clotting, fibrinolysis or complement cascades, but also inactivation of endogenous proteinase inhibitors by unspecific proteolysis due to granulocytic proteinases.

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