REPLACEMENT THERAPY OF ACQUIRED ANTITHROMBIN III DEFICIENCY IN EXPERIMENTAL NEPHROTIC SYNDROME

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Antithrombin III (AT) is the chief physiological inhibitor of serum proteinases of the hemostasis system, and for that reason an inborn or acquired deficiency of AT is accompanied by increased risk of thrombus formation. Clinical and experimental forms of nephrotic syndrome (NS) are accompanied by an acquired deficiency of the inhibitor, associated with loss of AT with the urine during proteinuria and with its utilization to inactivate enzymes of the clotting system in hypercoagulemia [13]. The fall of the plasma AT level correlates with the frequency of occurrence of thrombosis and embolism in NS [15]. Data on the use of AT preparations in clinical practice and experimental research are scanty. It has been shown, for instance, that injection of AT prevents the development of an experimental disseminated intravascular clotting (DIC) syndrome, considerably reduces the severity of disturbances of the hemostasis system and clinical manifestations in virus hepatitis and endotoxic shock, complicated by DIC, and reduces the frequency of thrombus formation in extracorporeal trunk vessels during hemodialysis on patients with renal failure. The aim of this investigation was to study the effect of AT on the state of the hemostasis system and on renal function during the development of chronic AT deficiency in NS.

EXPERIMENTAL METHOD

NS was produced in male albino rats weighing 180-200 g by the method in [1]. The animals were used in the experiments 1.5 months after injection of the immunogen. Protein concentration in the urine of the experimental animals, determined by the method in [3], was 274 ± 51 μg/ml (physiological proteinuria in the control was 20 ± 5 μg/ml). The urea concentration determined by a "Bio-La Test" kit (Chemapol, Czechoslovakia), was 15.3 ± 0.5 mmole/liter

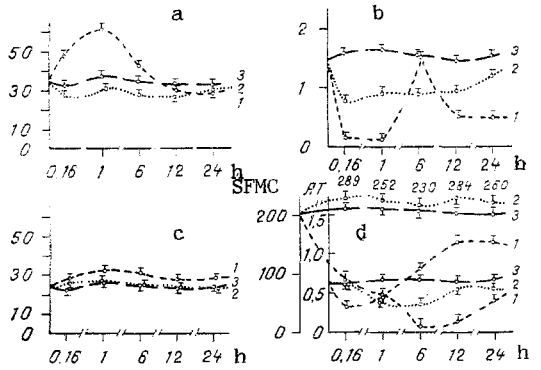

Fig. 1. Changes in parameters of hemostasis system after injection of AT and albumin into rats with NS. a) APTT (in sec), b) thrombin generation test (in NIH units/ml), c) thrombin time (in sec), d) AT activity (in U/ml) and SFMC concentration (in μg/ml). 1) Injection of bovine AT in a dose of 25 U/kg (1.25 mg/kg); 2) injection of BSA (1.25 mg/kg); 3) injection of 1 ml of 0.9% NaCl solution.

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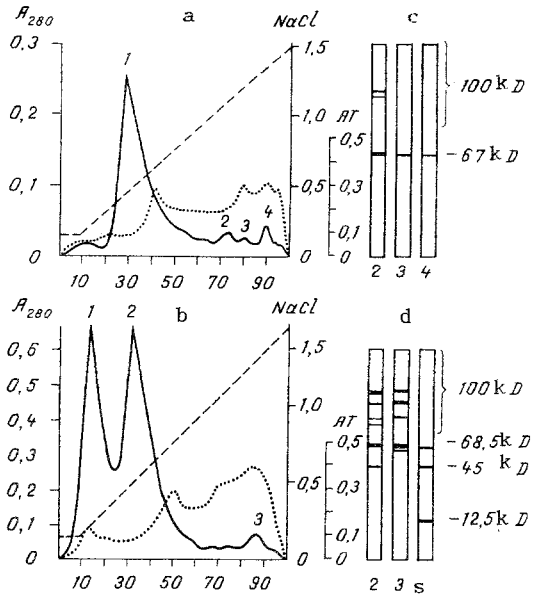

Fig. 2. Isolation of AT from blood plasma of rats with NS by affinity chromatography on heparin–sepharose before and after the end of AT injection. a) Chromatographic elution profile of blood plasma proteins of rats with NS from heparin–sepharose column (1.8 × 16 cm) by 0.1M Tris-HCl buffer, pH 7.4, containing 0.01M sodium citrate in an NaCl concentration gradient (0.15-1.5M); b) chromatographic elution profile of blood plasma proteins from rats with NS from heparin–sepharose column after administration of AT for 10 days under the same conditions; c) electrophoresis of AT from rats with NS: 2) component 2 with mol. wt. > 100, > 100, and 69 kilodaltons (kD), 3) component 3 with mol. wt. of 67 kD, 4) component 4 with mol. wt. of 67 kD; d) electrophoresis of AT from rats with NS after administration of AT for 10 days: 2) component 2 with mol. wt. > 100, > 100, > 100, > 100, 70, and 44 kD, 3) component 3 with mol. wt. > 100, > 100, > 100, 75, and 64 kD. s) Standards: albumin, ovalbumin, cytochrome C. Continuous line indicates A_{280}; dotted line shows AT activity (in U/ml); broken line represents NaCl concentration (in M).

compared with 6.31 ± 0.6 mmole/liter in the control. AT was obtained from bovine blood plasma by affinity chromatography on heparin–sepharose [11, 14], synthesized by the method in [4]. The final preparation contained 250 μg AT in 1 ml (activity 20 U/mg), it was homogeneous on polyacrylamide gel (PAG) during electrophoresis in the presence of sodium dodecyl sulfate (SDS), and its molecular weight was 65 ± 2 kilodaltons. One unit (U) of AT activity was taken to be the antithrombin activity of 1 ml of bovine blood plasma, determined by the method in [2]. The preparation was injected once a day for 10 days in a dose of 25 U/kg. In the control series of experiments (loading with heterologous protein) bovine serum albumin (BSA; from Reanal, Hungary) was injected into rats with NS in a dose of 1.25 mg/kg. The number of injections was the same as when AT was used. The preparations were injected into the jugular vein. Blood was taken from the same vein and stabilized with 3.8% sodium citrate solution in the ratio of 9:1. Blood for investigation was taken 10-15 min and 1, 6, 12, and 24 h after injection of AT, and 24 h after the end of the 10 daily injections of the preparation. The following parameters of the hemostasis system were studied: the activated partial thromboplastin time (APTT) using an "APTT-kit" (Reanal, Hungary), thrombin generation [7], thrombin time [5], and the concentration of fibrinogen and of soluble fibrin–monomer complexes (SFMC) [2]. Plasma AT activity was determined by the method developed by the firm Boehringer Mannheim (West Germany) on the chromogenic substrate Chromozym TH, with an "antithrombin-III kit." AT activity in the animals' urine was investigated by diffusion in thrombin–agarose [12]. AT was isolated from the blood plasma of rats with NS by chromatography on heparin–sepharose, before and after the end of injection of the preparation. The AT fractions were subjected to PAG electrophoresis in the presence of SDS [6].

TABLE 1. Parameters of the Hemostasis System and of Renal Function after Daily Injections of Albumin and AT for 10 Days into Rats with Experimental NS (M ± m)

Parameter	Experimental conditions		
	NS (n=13)	NS + BSA (n=10)	NS + AT (n=10)
APTT, sec	36,5±1,9	22,3±1,0	30,0±0,8
p	—	<0,001	<0,001
p_1	—	—	<0,01
Thrombin time, sec	23,4±0,3	23,4±0,4	27,8±0,6
p	—	>0,05	<0,001
p_1	—	—	<0,001
Thrombin generation test, NiH, units/ml	1,43±0,13	4,6±1,0	0,42±0,02
p	—	<0,001	<0,001
p_1	—	—	<0,001
AT, U/ml	0,56±0,04	0,4±0,05	1,0±0,03
p	—	<0,02	<0,001
p_1	—	—	<0,001
SFMC, µg/ml	199±11	245±28	70±13
p	—	>0,2	<0,001
p_1	—	—	<0,001
Fibrinogen,	4,38±0,3	5,16±0,7	4,23±0,3
p	—	>0,05	>0,05
p_1	—	—	>0,05
Urinary protein, µg/ml	274±51	710±101	430±45
p	—	<0,001	<0,02
p_1	—	—	<0,05
Blood urea, mmoles/liter	15,3±0,5	18,1±2,1	6,8±0,9
p	—	>0,05	<0,001
p_1	—	—	<0,001
Urinary AT, U/ml	0,1±0,05	0,15±0,03	0,2±0,03
p	—	>0,05	>0,05
p_1	—	—	<0,001

Legend. p_1) Significance of differences between values in rats with NS receiving and not receiving AT (NS and NS + AT).

EXPERIMENTAL RESULTS

The development of experimental NS was accompanied by activation of the clotting and depression of the anticlotting systems, revealed as lengthening of APTT by 32% ($p < 0.01$), and reduction of the thrombin-generating (by 57%, $p < 0.05$) and anticoagulant (by 15%, $p < 0.01$) activity of the plasma. Under these circumstances the concentration of molecular markers of thrombinemia (SFMC) was increased by 6.5 times, and the fibrinogen concentration increased by 65% ($p < 0.001$). Plasma AT activity was reduced by 43% ($p < 0.001$) and the level of the inhibitor in the urine reached 0.1 ± 0.05 U/ml. Slowing of prothrombinase formation by the endogenous route and lowering of the thrombin-generating activity of the plasma were connected with a combined decrease in the concentration of several factors of the endogenous route of blood clotting during NS caused by proteinuria [13].

The time course of changes in the parameters of the hemostasis system under the influence of AT was characterized by progressive inhibition of prothrombinase formation for 1 h after injection of the inhibitor (Fig. 1a). However, after 6 h the rate of prothrombinase formation was already close to its initial level, and 12-24 h after injection of AT its formation was found to be accelerated. During the first hour, under the influence of AT the thrombin generating activity of the plasma fell regularly (Fig. 1b). At the 6th hour of the experiment the ability of the plasma to generate thrombin was close to its initial level, but 12-24 h after injection of the preparation, it was observed to fall again. The SFMC concentration, reflecting the dynamics of thrombin production in vivo, was depressed at all times of observation and reached a minimum 6 h after injection of AT (Fig. 1d). Under the influence of AT a stable increase of the anticoagulant activity of the plasma, reflected in the thrombin time, was observed for 24 h (Fig. 1c). Hexogenous AT caused fluctuations of plasma

AT activity: 10 min after its injection activity of the inhibitor fell by almost a half below its initial level, which it did not regain until the end of the first hour of the experiment (Fig. 1d), after which it increased progressively, to twice the original value, at which it still remained 24 h after injection.

The reduction of plasma AT activity during the first hour after injection of the exogenous inhibitor appears paradoxical. However, taking into consideration the signs of excessive thrombin production in the experimental animals and the high affinity of thrombin for vascular endothelium (dissociation constant 10^{-8} M) [8], it can be tentatively suggested that the enzyme binds with the vessel wall but, at the same time, preserves its ability to interact with AT, to form enzyme-inhibitor complexes [10]. According to data in the literature [9], the equimolar thrombin–AT complex binds an additional one or two molecules of the inhibitor, which leads to dissociation of the high-molecular-weight thrombin–AT associate from its binding with the matrix and to its release into the blood stream. That these hypotheses are correct is shown by data obtained during isolation of AT from the blood plasma of rats with NS, before and after injection of AT. It was shown that AT fractions from rats with NS contain high-molecular-weight complexes of AT with thrombin (mol. wt. $\geq$ 100 kD), with lower affinity for immobilized heparin than native AT (Fig. 2a, c). As a result of injection of AT into rats with NS the number of circulating AT complexes with serum proteinases of hemostasis in the systemic circulation increases considerably (Fig. 2b, d).

Replacement therapy of the acquired AT deficiency for 10 days in experimental NS restored some of the parameters of the hemostasis system to normal and reduced the parameters of hypercoagulation (Table 1). Compared with values characteristic of animals with NS and not receiving AT, prothrombinase formation by the endogenous route was accelerated and the thrombin generating activity of the plasma and the SFMC concentration were reduced. Activity of plasma anticoagulants and of AT was increased. Under the influence of AT the filtration function of the kidneys was not restored to normal (Table 1), as shown by the increase in proteinuria and urinary AT activity of the experimental animals. However, the excretory function was restored in this case, as shown by the fall in the blood urea.

Activation of the clotting system and excessive thrombin production in animals with NS caused disturbances mainly of the excretory function of the tubular system of the kidneys. By inhibiting activity of the enzymes of hemostasis, AT effectively corrected disturbances of renal excretion of metabolites.

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