# ROLE OF THE ANTITHROMBIN III—HEPARIN COMPLEX IN THROMBIN NEUTRALIZATION IN HUMAN AND ANIMAL BLOOD

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It was shown previously [6] that intravenous injection of tissue thromboplastin (0.4 ml/200 g) into animals leads to rapid, avalanche-like generation of thrombin in the blood stream, in a concentration of $5 \cdot 10^{-2}$ U/ml, in the course of 30-50 sec after the injection. Meanwhile, signs of activation of the anticlotting system are observed in the blood of experimental animals in response to the formation of the clotting enzyme, namely an increase in the anticoagulant and fibrinolytic potential of the blood [2], leading to complete neutralization of the thrombin formed 15 min after injection of thromboplastin. The main humoral agents in the blood of animals responsible for neutralizing thrombin are heparin (H), its complexes with blood proteins and amines, antithrombin III (AT III), $\alpha_2$-macroglobulin, and certain other protease inhibitors.

In the course of the reaction of the anticlotting system to thrombin, appearing in the blood stream, AT III activity also falls quickly and considerably to begin with, and this confirms its direct involvement in neutralization of the clotting enzyme [7]. In the presence of H the reaction of thrombin neutralization with AT III is considerably intensified, as shown by experiments in vitro [13] and in vivo [8]. An AT III—H complex was obtained in vitro which, besides anticoagulant activity, also possessed marked nonenzymic fibrinolytic properties [4], present in complexes of H with several blood proteins [5].

It can thus be postulated that in vivo the AT III—H complex plays the role of an important humoral agent of the anticlotting system, whose action is directed toward neutralization of thrombin and strengthening of the nonezymic fibrinolytic potential of the blood.

The aim of this investigation was to determine activity of the AT III-H complex in the blood stream of animals after activation of the function of the anticlotting system by intravenous injection of tissue thromboplastin, and also in the blood of children suffering from traumatic shock during the period of activation of function of the anticlotting system.

## EXPERIMENTAL METHOD

Noninbred albino rats weighing 180-200 g were used. Tissue thromboplastin was injected intravenously into the rats of group 1 in a dose of 0.4 ml/200 g body weight. The corresponding volume of physiological saline was injected intravenously into the animals of group 2. In clinical investigations blood samples were taken from children aged 4-10 years with traumatic shock of the I-III degree resulting from automobile accidents, and sustaining fratures, hemorrhage, and crushing of the internal organs.

Tissue thromboplastin was prepared from rat brain tissue in the form of a suspension (1 g of brain tissue was ground in a mortar with 10 ml of 0.85% NaCl solution), which was centrifuged before use in order to sediment solid particles.

Blood for analysis was taken from the animals' jugular vein in a volume of 3 ml into sodium citrate in the ratio of 9:1, 10 min after injection of thromboplastin, and it was centrifuged at 1333g for 15 min to remove platelets from the plasma. Blood was taken from the children on admission, before transfusions for shock treatment, and 2-3 days later, during

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TABLE 1. Blood Clotting Indices and NEFA of AT III–H Complex Isolated from Animals' Blood after Intravenous Injection of Tissue Thromboplastin (0.4 ml/200 g) during Activation of Function of Anticlotting System (M ± m)

| Experimental conditions | Number of animals | Plasma | | | | | Anticoagulant activity of AT III-H complex, sec | NEFA of AT III-H complex, mm² |
|---|---|---|---|---|---|---|---|---|
| | | fibrinogen mg% | thrombin activity, sec | AT III, % | TFA, mm² | NEFA, mm² | | |
| Administration of thromboplastin (experiment) | 18 | 121±13* | 321±18* | 66,0±4,8* | 88,0±5,3* | 59,4±4,6* | 15,8±0,5 | 41,5±5,1* |
| Administration of physiological saline (control) | 15 | 293±19 | 438±23 | 104,0±4,1 | 54,0±4,2 | 28,7±2,1 | 15,7±0,4 | 22,5±2,7 |

Legend. *$p < 0.05$.

treatment. The AT III–H complex was isolated from plasma obtained from the individual blood samples of animals and children. For this purpose 1.5 ml of plasma was heated to 55°C for 3-4 min to remove fibrinogen. From the supernatant remaining after sedimentation of fibrinogen, prothrombin and factors of the prothrombin complex were removed by adsorption on $BaSO_4$ (as used in x-ray investigation) powder, after mixing for 30 min. After removal of the precipitate euglobulins were removed from 1 ml of each specimen of supernatant, after which the residual supernatant was acidified to the pH for precipitation of the complex (4.6-4.8) [4], incubated at 4°C for 20-30 min to consolidate the precipitate, centrifuged at 1333 g for 20 min, and the almost invisible residue of the complex thus obtained was dissolved in phosphate buffer, pH 7.4-7.8.

The anticoagulant activity of the AT III–H complex was determined by the usual method (reaction: 0.1 ml of a solution of the complex + 0.1 ml of a 0.2% solution of fibrinogen + 0.1 ml of a solution of thrombin), nonenzymic fibrinolytic activity (NEFA) was determined as in [3] by application of 0.025 ml of a solution of the complex, in the presence and in the absence of ε-aminocaproic acid, to unstabilized fibrin plates, plasmin activity was determined on standard fibrin plates by the method in [9], H by the method in [14], and AT III by the method in [12]. Electrophoretic analysis of the complex was undertaken by the method in [10] in polyacrylamide gel in the presence of sodium dodecylsulfate.

The concentration of fibrinogen [1] in samples of blood plasma from the animals and children [1], the total fibrinolytic activity (TFA) and NEFA of plasma [3], AT III activity (by the method in [12]), and the free thrombin formation time [11] were determined.

## EXPERIMENTAL RESULTS

It will be clear from Table 1 that 10 min after intravenous injection of tissue thromboplastin weak free thrombin activity ($5 \cdot 10^{-5}$ U/ml) was observed in the animals' blood (the fibrin aggregation time in the reaction mixture was 321 ± 18 sec), the fibrinogen concentration was reduced (by 2.4 times compared with the control), plasma TFA and NEFA were considerably increased (by 1.5 and 2 times, respectively), and AT III activity fell. This is evidence of activation of the function of the anticlotting system, as a result of which activity of the clotting enzyme at this period of the experiment was already below its initial high level at the period of maximal thrombin generation during the first minute after injection of tissue thromboplastin, namely $5 \cdot 10^{-2}$ U/ml [6] (the fibrin aggregation time under these circumstances was 158 ± 14 sec). NEFA of the AT III–H complex, isolated from the blood of animals receiving thromboplastin, was twice as high as in the control animals, receiving an injection of physiological saline. The AT III content in the composition of the AT III–H complex isolated from the blood of the experimental animals was also much higher (21.0 ± 4.1%) than in the complex isolated from blood of normal rats (3.5 ± 1.8%). Plasmin activity could not be found in any of the complexes. The H content in the experimental samples of complexes was 0.070 ± 0.001 μg/ml, which also was higher than its level in specimens of complexes isolated from the blood of animals of the control group (0.053 ± 0.001 μg/ml).

After dissociation of the complexes isolated from the blood of animals of both groups by alkalifying the solution of the complex to pH 8.3, thrombin activity was found in them. For instance, whereas before alkalification the activity of the complex was 39 sec, after the change of pH the thrombin time was shortened to 29 sec. In the control reaction of the mix-

ture thrombin activity before alkalification was 25.5 sec, compared with 27 sec after alkalification. Electrophoretic analysis of the dissociated complexes isolated from the blood of the experimental animals after injection of tissue thromboplastin revealed two protein fractions with mol. wt. of 60 and 40 kilodaltons (kD), corresponding to the molecular weights of AT III and thrombin.

The results are thus evidence that the AT III—H complex binds circulating thrombin with the formation of a triple AT III—H—thrombin complex. This confirms earlier data obtained in vitro [14]. In a state of activation of function of the anticlotting system in animals after intravenous injection of tissue thromboplastin, NEFA of complexes isolated from the blood was higher than the activity of complexes isolated from the blood of normal animals, and this was combined with other features of activation of the function of the anticlotting system. The anticoagulant activity of the isolated complexes, however, was only slightly increased (15.8 ± 0.5 sec) compared with that of the control reaction mixture, containing an equal volume of phosphate buffer instead of the solution of the complex (10 ± 0.1 sec, $p < 0.001$), which was evidently due to the presence mainly of the triple complex in the isolated complexes. It was shown previously that the pH of the precipitation of the AT III—H complex is the same as that of the triple complex, and that when thrombin is incorporated into the AT III—H complex the anticoagulant activity of the complex is reduced or completely abolished [4].

During investigation of blood samples from children with traumatic shock, taken on the first day after admission, in the torpid phase of shcok before the beginning of combined antishock infusion therapy, increased activity of free thrombin was found in the blood (the fibrin clot formation time in the patients averaged 280 sec compared with 455 sec in healthy subjects), activity of AT III was reduced (66 and 112% on average, respectively), the fibrinogen concentration was reduced (220 and 340 mg %), and the TFA of the plasma (57 and 39 $mm^2$) and nonenzymic fibrinolysis (33 and 20 $mm^2$) were increased. All these parameters reflect activation of function of the anticlotting system, taking place in the early stages of development of pathology. AT III—H complexes isolated from the patients' blood during this period of observation showed the presence of high NEFA (on average 66 $mm^2$).

Activity of complexes isolated from blood samples taken after 2-3 days of antishock treatment was low (on average 26 $mm^2$), and was combined at this period of development of pathology with hypercoagulation and depression of function of the anticlotting system (the fibrinogen concentration was increased on average to 610 mg %, and free thrombin activity was increased — the time of formation of fibrin clots averaged 190 sec).

The experimental and clinical data are evidence that during activation of the function of the anticlotting system the role of the AT III—H complex in prevention of the action of thrombin generated in the blood stream is realized both through its participation in the formation of an AT III—H—thrombin complex and neutralization of the clotting enzyme, and through an increase in the nonenzymic fibrinolytic potential of the blood plasma.

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