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INDUCTION OF ANTITHROMBIN III (AT III) ANTIBODIES BY IMMUNIZATION WITH SYNTHETIC PEPTIDES

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The primary structure of AT III was examined in respect of potential antigenic sites. The topics were the determination of the hydrophilicity, hydropathy, acrophilicity and the propensities for alpha-helices, ß-turns and ß-sheets. The peptides AT III 21 - 34, 21 - 42, 129 - 140, 226 - 240 and 343 - 363 were synthesized using the solid phase peptide synthesis methode. The subsequent purification of the crude peptides was achieved by h.p.l.c. or by ion exchange chromatography. The peptides were coupled to keyhole limpet hemocyanine (KLH) via thioether bonds. Antisera against KLH-peptides were raised in rabbits (n = 25) and tested with AT III-coated polystyrene tubes; bound antibodies were detected with anti-rabbit-IgG-peroxidase. Obtained antisera were further purified by immunoadsorption using immobilized AT III. Polystyrene tubes were coated with purified peptide antibodies and binding of AT III was studied with enzyme immunoassay technique (EIA) using anti-AT III-peroxidase.

As a result, immunoreactivity of rabbit antisera against synthetic peptides of AT III could be achieved. The obtained antibodies against the individual synthetic peptides as well as their mixtures exhibited specific binding to AT III when tested with EIA.

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IN VITRO REACTION MODELS OF THROMBIN AND ITS PHYSIOLOGICAL INHIBITOR ANTITHROMBIN III IN THE PRESENCE OF HEPARIN.

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Antithrombin III (AT III) neutralizes thrombin and other serine proteases of plasma coagulation system by forming a stable 1:1 covalent complex. The inhibition rates are greatly increased by the potent catalyst heparin. The catalytic mechanism of heparin was studied in the presence of dextran sulfate (DS), a thrombin-binding sulfated polysaccharid. DS did not influence the reaction of AT III with heparin and the amidolytic activity of thrombin, but preincubation with thrombin could inhibit the catalytic activity of heparin in the reaction of thrombin with AT III. We conclude that the reaction of heparin with enzyme and inhibitor, thus forming a ternary complex, is necessary for its catalytic activity.
It is known that heparin also converts AT III from an inhibitor to a substrate for thrombin in a dose dependent manner. By cleavage of the reaction site bound Arg(385)-Ser(386) an AT III-fragment (MG 50000 d) occurs, which has a decreased affinity to heparin and does not inhibit F IIa. At physiological ionic strength we have only measured a small percentage of AT III-proteolysis (4%, 1 U/ml Hep). The extent of AT III-fragment formation could be enhanced by lowering the ionic strength (max 44%, 1 U/ml Hep., I=0,02).

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THE ROLE OF HEPARIN CHARGE DENSITY IN THE ANTITHROMBIN III-DEPENDENT AND ANTITHROMBIN III-INDEPENDENT INACTIVATION OF THROMBIN.

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The dependence of the anticoagulant properties of heparin upon charge density may reflect structural factors that are important in anti-thrombin effect. We have previously demonstrated that in the absence of antithrombin III (AT III) unfractionated heparin inhibits the catalytic effect of thrombin upon platelet activation. In the present study we evaluated the thrombin-binding properties of heparin fractions obtained by ion-exchange chromatography on DEAE-Sephacel. We found that these fractions were able to bind to thrombin with an affinity that increased with their charge density. This was shown by their inhibitory effect in the absence of AT III on thrombin-catalyzed platelet factor Va formation and by the ability of active site blocked thrombin to prevent the heparin-dependent inactivation of thrombin by AT III. However, their increase in charge density and thus affinity for thrombin was found to go along with an increase in AT III-binding sites, as measured by the heparin-dependent increase of the intrinsic fluorescence of AT III. Moreover all heparin fractions showed the same specific antithrombin activity when the molar concentration of AT III-binding heparin was taken into account. We also investigated the thrombin-binding properties of two heparin fractions obtained by affinity chromatography on AT III-Sepharose. The AT III low affinity fraction was practically devoid of any inhibitory effect on the rate of the thrombin-catalyzed factor Va formation, indicating a low, if any, affinity for thrombin. In contrast the AT III-independent inhibition of thrombin was completely recovered from the AT III high affinity fraction. In addition, we also established that when the heparin fraction from the DEAE-Sephacel column, with the lowest charge density and very low in AT III binding material, was modified by the incorporation of sulfate groups so as to achieve a higher charge density, it obtained a higher affinity for thrombin but this modification caused the loss of half the AT III binding sites. In conclusion, it is apparent that fractionation of crude heparin on a DEAE-Sephacel column or on an AT III-Sepharose column does not result exclusively in a separation of either the thrombin-binding or the AT III-binding heparin fractions.

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PRODUCTION AND CHARACTERISATION OF A MONOCLONAL ANTIBODY AGAINST HUMAN ANTI-THROMBIN III.

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To study the role of anticoagulants, particularly antithrombin III (AT III) and heparin, on the activation of coagulation by monocytes/macrophages which have been stimulated with a soluble lymphocyte activation product, macrophage procoagulant inducing factor, we have prepared monoclonal antibodies (MAbs) to human AT III.

In fusion experiments, in contrast to wells containing peritoneal feeder cells, positive hybrids were only found in wells containing medium conditioned by the macrophage cell line J774 (Rathjen and Geczy, 1986). Of 5 hybrids which initially produced antibody, only one hybrid, showed stable Ab production. The MAb, designated 22/23, was not cross-reactive with either α_1 antitrypsin or ovalbumin and did not inhibit the biological activity of AT III in chromogenic assays which measured inhibition of thrombin and Factor Xa by AT III. An immunoadsorbent prepared using MAb 22/23 depleted AT III activity from a purified AT III preparation. Reduction and alkylation of the disulphide bonds of the protein portion of AT III completely abbrogated MAb binding indicating that the native configuration of AT III was important. Isoelectric focussing of AT III, followed by transfer of the focussed protein to nitrocellulose by diffusion and probing with MAb 22/23, revealed at least 8 bands in the region of pH 5.2 to 5.85. Coomassie blue staining of a gel run in parallel showed 9 bands in this region. The MAb provides a useful tool for the detection of AT III on both cultured cells (bovine aortic endothelial cells are positive by immunofluorescence) and tissue sections.

Rathjen, D.A. and Geczy, C.L. Hybridoma. 5: 255-261 (1986)