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NEOANTIGENIC EXPRESSION IN ENZYME·INHIBITOR COMPLEXES A MEANS TO DEMONSTRATE ACTIVATION OF ENZYME SYSTEMS

DESIRE COLLEN and FRANS DE COCK

Center for Thrombosis and Vascular Research, Department of Medicine, University of Leuven (Belgium)

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Summary

Human thrombin·antithrombin III and plasmin·antiplasmin, two enzyme·inhibitor complexes composed of four different molecules, contain antigenic structures not present in the parent molecules, which can be directly quantitated in plasma with the use of non-cross-reacting antisera.

It is anticipated that this neoantigenic expression is a more general phenomenon, which could provide a simple means of measuring activation of enzyme systems in biological fluids.

Several plasma enzyme systems, such as the coagulation, fibrinolytic, complement and kallikrein-kinin system, contain both zymogens and enzyme inhibitors. Activation of these systems results in formation of active enzymes which have a short lifespan in the blood as they become bound to and neutralized by their inhibitors. We have raised the question whether enzyme-inhibitor formation could be associated with the emergence of structural or conformational alterations which render these complexes antigenically distinct from the precursor molecules. Immunochemical quantitation of such neoantigens would then constitute a means of measuring activation of these enzyme systems. The generation of neoantigenic expression in protein molecules following antigen-antibody complex formation [1] or upon denaturation or enzymatic degradation [2] has already been described.

Blood coagulation results in the formation of thrombin which is mainly neutralized by antithrombin III (heparin cofactor). Fibrinolysis is the result of activation of plasminogen, the formed plasmin being neutralized by a recently discovered fast-reacting inhibitor which we have called antiplasmin [3,4].

Address for correspondence: D. Collen, Center for Thrombosis and Vascular Research, Gasthuisberg - Onderwijs en Navorsing, University of Leuven, Herestraat 49, B-3000 Leuven, Belgium.

The thrombin·antithrombin III complex was formed in reptilase-defibrinated human plasma by activation of the coagulation system via the intrinsic pathway. We isolated the thrombin·antithrombin III complex from this mixture by affinity chromatography on insolubilized heparin and gel filtration [5]. The resulting material displayed two to four bands on sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Rabbits were immunized with this material using conventional procedures. Specific antisera against neoantigens in the complex were obtained by multiple step absorption with human plasma in the presence of the anticoagulant heparin and the serine protease inhibitor *p*-nitrophenyl-*p'*-guanidinobenzoate (*p*-NPGB) to prevent formation of the enzyme·inhibitor complex in human plasma during incubation with rabbit serum. The specific antibodies were then isolated by adsorption on insolubilized thrombin·antithrombin III complex and elution with 3 M potassium thiocyanate.

The plasmin·antiplasmin complex was formed in human plasma by activation with streptokinase. The plasmin·antiplasmin complex was isolated from this mixture by affinity chromatography on lysine-agarose and gel filtration [3]. The resulting material displayed two bands on SDS polyacrylamide gel electrophoresis with molecular weights of 120 000 and 140 000. Rabbits were immunized with this material and the antisera rendered specific for the complex by multiple step absorption with human plasma and with purified plasminogen in the presence of the fibrinolytic inhibitor aprotinin (Trasylol®, Bayer) and *p*-NPGB. The specific antibodies were then isolated using insolubilized plasmin·antiplasmin complex as immunosorbent.

Fig. 1 (upper half) shows that rabbit antisera raised against thrombin·antithrombin III complex contain antibodies to several components present in human plasma, but reveals at least one additional intense precipitin line with human serum. The purified complex-specific antibodies do not react (or very poorly) with human plasma but form at least one intense precipitin line with human serum.

Fig. 1 (lower half) shows that the rabbit serum raised with plasmin·antiplasmin complex reacts with two components in human plasma with β_2-mobility (plasminogen) and α_2-mobility (antiplasmin). In urokinase-activated plasma these components have greatly diminished or disappeared and a new component with β_1-mobility has emerged. The purified, complex-specific antibodies only react with two immunologically identical components in urokinase-activated plasma and not with normal plasma.

Fig. 2 shows electroimmunoassays of dilutions of serum, plasma and urokinase-activated plasma in agarose gel containing specific antibodies against the thrombin·antithrombin III or plasmin·antiplasmin complex. The antibodies appear to react only with their specific complexes and not with fresh plasma or plasma in which the other complex has been formed.

An agglutination technique, using latex particles coated with the purified complex-specific antibodies was developed to quantitate both complexes in plasma [5,6]. The purified complexes caused agglutination of the particles in concentrations exceeding 0.2—0.3 mg per liter. The purified precursors (plasminogen and antiplasmin in the test for plasmin·antiplasmin complex and prothrombin and antithrombin III in the test for thrombin·antithrombin

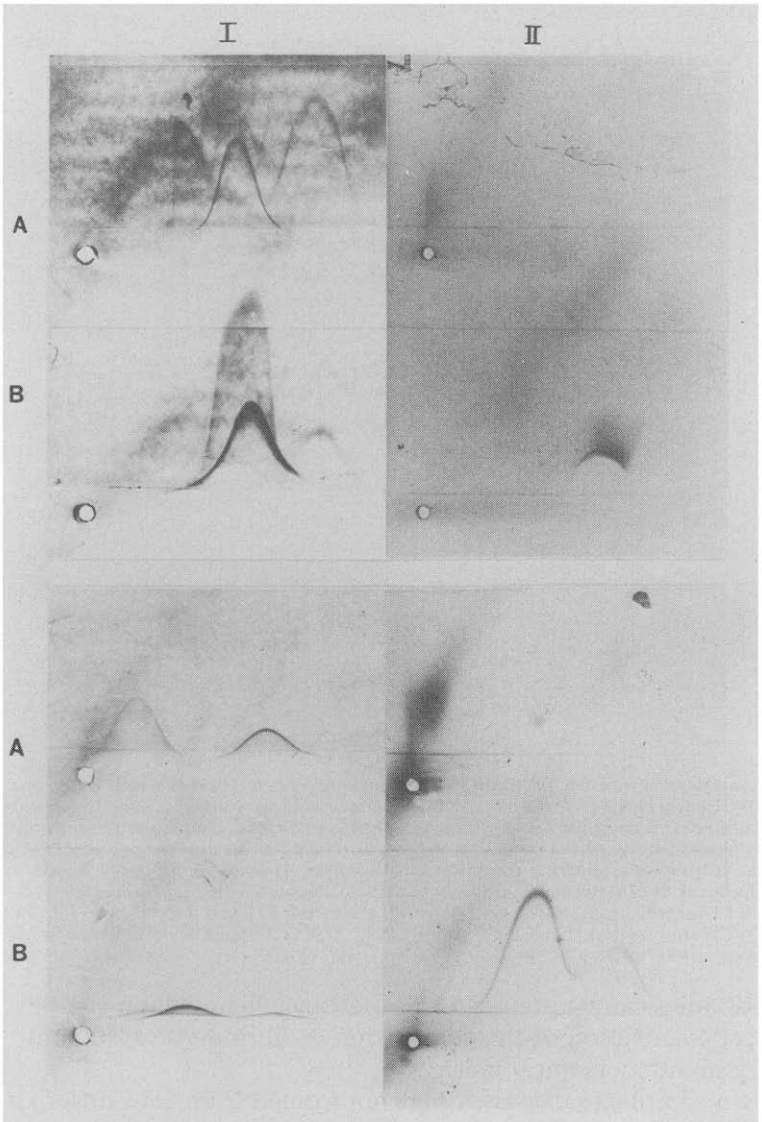

Fig. 1. Crossed immunoelectrophoresis: upper half, with antisera against thrombin•antithrombin III; lower half, with antisera against plasmin•antiplasmin. I, Unabsorbed antiserum; II, purified antibodies against neoantigens in the complex. A. Fresh human plasma. B. Activated human plasma (serum in the upper half, urokinase-activated plasma in the lower half).

III complex) were at least 100 times less reactive than the purified complexes. Preliminary experiments with radioimmunoassay techniques gave similar results. The latex agglutination tests have successfully been applied to the diagnosis of in vivo activation of the coagulation and/or fibrinolytic systems in human blood [5,6].

All these data indicate that both the thrombin•antithrombin III and plasmin•antiplasmin complex contain neoantigenic structures to which non-

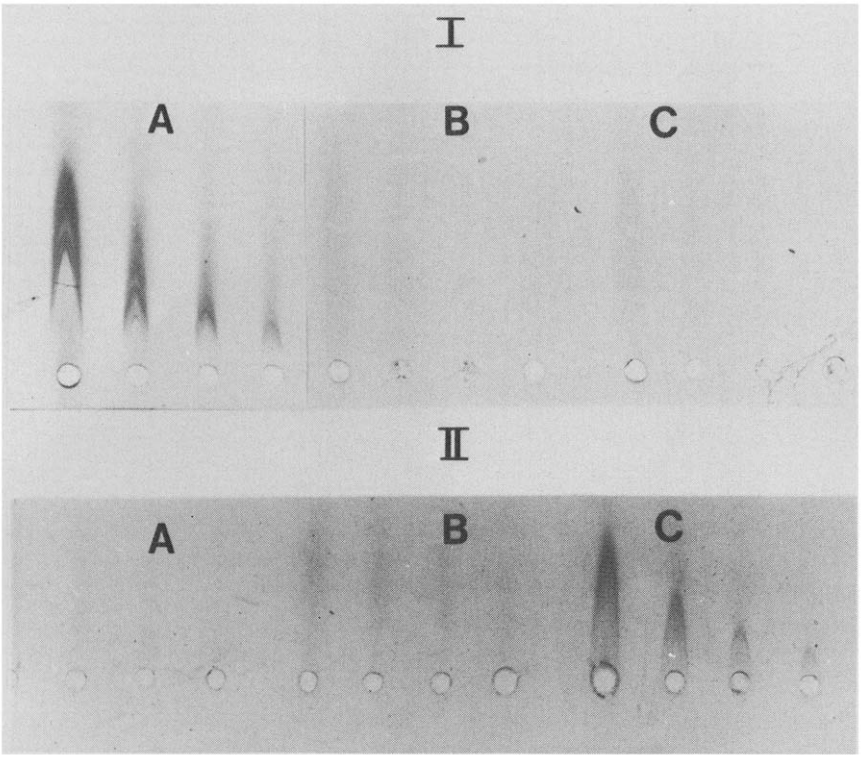

Fig. 2. Electroimmunoassay with purified antibodies against neoantigens. A, Human serum (dilutions 1/2, 1/4, 1/8, 1/16); B, Human plasma; C, Human plasma activated with urokinase. I, Specific antibodies against the thrombin•antithrombin III complex; II, specific antibodies against the plasmin•antiplasmin complex. The heterogeneity of the precipitin rockets observed on electroimmunoassay of serum in a gel containing antibodies against the thrombin•antithrombin III complex probably results from the fact that complexes of other coagulation factors with antithrombin III (e.g. Factor Xa•antithrombin III and Factor IXa•antithrombin III) are formed in the serum [7] and were also present in the purified material for immunization. This would suggest that these complexes contain neoantigens which are different from one another.

crossreacting antibodies can be prepared and that this phenomenon can be used to demonstrate activation of the coagulation or fibrinolytic system in human plasma by immunochemical means.

Since the two complexes discussed here are formed from four different proteins, it seems probable that other enzyme•inhibitor complexes also contain neoantigenic structures, and that immunochemical quantitation of these may constitute a general method for demonstrating activation of enzyme systems in biological fluids containing both zymogens and enzyme inhibitors.

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