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EVIDENCE FOR AN IMMUNE MECHANISM OF HEMOSTATIC ENZYME CONTROL

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The writers previously suggested the existence of an autoimmune mechanism of control of activated enzymes in the blood stream [1, 2]. It was postulated that activation of an enzyme (proteolysis or conformational changes) is accompanied by the appearance of previously latent antigenic determinants, which act as the afferent signal for triggering immune mechanisms of enzyme inhibition and elimination.

This paper describes an attempt to isolate antibodies against modified enzymes of the hemostasis system from the blood of unimmunized animals.

EXPERIMENTAL METHOD

Bovine blood stabilized with 2% potassium oxalate solution was used. Plasma was coagulated with 1% $CaCl_2$ solution, the resulting fibrin was removed, and the serum thus obtained served as the source of active Stuart-Prower (Xa) factor (fXa). The factor was isolated by ion-exchange chromatography on DEAE-Sephadex A-50 (from Pharmacia, Sweden) using stepwise elution gradients of phosphate buffer (0.05M, pH 7.0; 0.2M, pH 7.0; 0.45M, pH 8.0). The fraction eluted with the last gradient of buffer was rechromatographed 3 times, desalted on Acrilex P-60, or dialyzed against water and lyophilized. The resulting preparation of fXa degraded BAME (from Reanal, Hungary) converted prothrombin (factor II, abbreviated to FII) into thrombin (factor IIa — fIIa) in a system of fII + fXa + Ca^{++}, and was homogeneous in polyacrylamide gel. Prothrombin was isolated from bovine plasma by the method in [5]. Thrombin (from the Kaunas Research Institute of Epidemiology, Microbiology, and Hygiene) was additionally purified by gel filtration on Sephadex G-100 (from Pharmacia). A 0.45% solution of NaCl, pH 7.2, was used as eluting field. Protein peaks with maximal enzyme activity in a system of fIIa + fibrinogen = fibrin, were pooled and lyophilized.

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TABLE 1. Titers and Crossed Reaction

Obtained on undermentioned sorbents	From γ-fraction of plasma	From γ-fraction of plasma
On fXa-Sepharose	1/16	1/4
On fIIa-Sepharose	1/32	1/4
On fXa-Sepharose against fIIa	1/8	1/4
On fIIa-Sepharose against fXa	1/8	1/8
γ-Fraction before elution against fXa	1/16	1/1
γ-Fraction before elution against fIIa	1/8	1/1
γ-Fraction after elution against fXa	0	0
γ-Fraction after elution against fIIa	0	0

Antibodies against the immobilized enzymes were isolated from the γ-fraction. This condition was dictated by the need to free the serum proteases from known inhibitors (antithrombin III, Cl-inhibitor, L_2-macroglobulin, L_1-antitrypsin). The γ-fraction was obtained from bovine serum or plasma by salting out with ammonium sulfate (40% saturation), desalted on Acrilex P-60, and additionally purified on DEAE-Sephadex A-50 in 0.02 M sodium-phosphate buffer, pH 6.6. The isolated enzymes (100 mg of each) were immobilized with Sepharose 4B (from Pharmacia), activated with cyanogen bromide (3 g of each) in 1 M $NaHCO_3$ in 0.5 NaCl buffer, pH 8.3. The mixture was spread on a shaker for 2 h at room temperature. Unbound protein was then washed off with combining buffer and the remaining reactable groups were blocked by GSA (from Reanol) or glycine (from Sigma, USA), 30 mg of each per column. After mixing on a shaker for 2 h at room temperature, the gel was washed successivly with 1 M $NaHCO_3$ in 0.5 M NaCl, pH 8.3; 0.1 M sodium-acetate buffer in 1 M NaCl, pH 4.0; 0.1 M borate buffer, pH 8.0; 0.005 M Tris buffer in 0.15 M NaCl until complete disappearance of protein from the eluates. The antienzymatic activity of the immunoglobulins (Ig) isolated on fXa-Sepharose was assayed as follows: fII in a volume of 0.1 ml (1 mg/ml) + fXa (1 mg/ml) + hemolysate + 1% $CaCl_2$ were mixed in a test tube. In the control 0.2 ml of 0.85% NaCl and in the experiment 0.2 ml of the isolated Ig was added to this system. After incubation for 2 min in a water bath at 37°C, 0.2 ml of a 0.4% solution of fibrinogen was added and the velocity of its conversion into fibrin was estimated. In another test system the prothrombin and fibrinogen were replaced by 0.1 ml of donor's plasm. Antithrombin activity of the Ig isolated on fIIa-Sepharose sorbent was determined in a system of fIIa + 0.2 ml 0.85% NaCl or 0.2 ml Ig. After incubation for 2 min in a water bath the fibrinogen or plasma was added. Activity of the isolated Ig was recorded in the passive hemagglutination test (PHT).

EXPERIMENTAL RESULTS

In our view, the content of Ig against modified enzymes differs in plasma and serum. In the latter, on account of fibrin formation, the Ig bind with and are utilized by activated procoagulants. Accordingly the Ig were isolated from the γ-fraction of both plasma and serum. The γ-fraction of plasma or serum, in a volume of 25 ml, was passed through 10 ml of residue of fXa- or fIIa-Sepharose. The antienzymatic activity was determined in samples of the γ-fraction after passage through the column and the PHT was performed. To remove unbound proteins the fXa- and fIIa-Sepharose were washed with combining buffer until complete disappearance of protein. Desorption of the Ig was carried out with HCl-glycine buffer, pH 2.2. The resulting eluates were immediately neutralized to pH 7.2 with 1M K_2HPO_4 and then dialyzed for 16 h against distilled water. The yield of Ig was: for the γ-fraction of plasma on fXa-Sepharose 0.06 mg/ml; on fIIa-Sepharose 0.06 mg/ml; from the γ-fraction of serum 0.05 and 0.03 mg/ml respectively.

The isolated preparations had inhibitory activity against enzyme. For instance, in a system of 0.1 ml fXa + 0.2 ml (incubation 2 min) + 0.1 ml plasma the inhibitory effect of the Ig isolated from the γ-fraction of plasma on fXa-Sepharose reached 20%. The Ig obtained from plasma on fIIa-Sepharose in a system of 0.1 ml fIIa + 0.2 ml (incubation 2 min) + 0.1 ml plasma increased the reaction time by 50%. In analogous systems Ig isolated from serum on fXa- and fIIa-Sepharose exhibited an effect of 17 and 12%, respectively.

Reduction of the effect of inhibition by Ig isolated from serum was probably due to their utilization in the process of fibrin formation.

The effect of inhibition by Ig isolated from serum on fXa-Sepharose in pure systems: 0.1 ml fXa + 0.2 ml Ig + 0.1 ml fII + 0.1 ml hemolysate + Ca^{++} (incubation 2 min) + fibrinogen was 26%. The Ig from the γ-fraction of serum, isolated on fIIa-Sepharose, inactivated thrombin by 9% in a pure system.

The isolated preparations on fXa- and fIIa-Sepharose gave a crossed inhibitory effect, which was between limits of 5 and 10%.

The γ-fraction exhibited no effects of inactivation of fXa and fIIa after passage through the column.

As Table 1 shows, the titers of antibodies against modified enzymes were considerably lower in serum than in plasma. This finding confirms yet again the probability that antifactors of immunoglobulin nature are used up in the course of fibrin formation. This fact under discussion is confirmed in [3], where autoantibodies against thrombin were found by Ouchterlony's method in the plasma of different animals and their disappearance in the serum was observed.

Meanwhile crossed reactions were found in the present experiments, possibly indicating the presence of common antigenic determinants in active factors of the prothrombin series; similar data were published previously [4]. This fact is extremely important. It can be postulated that Ig "common" to all factors of the prothrombin complex (IIa, VIIa, IXa, Xa) is present, as is confirmed, in particular, by crossed inhibition reactions of fIIa which we found in the PHT.

The data described in this paper thus confirm the presence of an immune mechanism controlling enzyme homeostasis.

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