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DETECTION OF AUTOANTIBODIES AGAINST BLOOD CLOTTING FACTORS BY PASSIVE HEMAGGLUTINATION TEST AND ELISA

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In the writers' view [4] a physiological immune mechanism of regulation of enzyme homeostasis in the blood clotting system exists in healthy humans and intact animals. Under these circumstances the procoagulant enzyme, unlike the proenzyme (inactive form of the procoagulant) is able to give an autoantigenic stimulus and to induce the formation of autoantibodies. These latter inhibit and eliminate the active procoagulant, and so take part in the creation of the general anticoagulant potential of the body. Experimental proof has been obtained of this hypothesis. For instance, immunoglobulins belonging to the IgG class and possessing anticoagulant activity have been isolated from the gamma-fraction of human and bovine plasma [9], and immunocompetent cells realizing an acceptor function relative to thrombin and factor Xa [8] have also been discovered in the blood stream. It has also been found that coagulopathies arising in immunodeficiencies both experimentally and clinically can be partly explained by a fall in the level of anticoagulant antibodies [3, 5].

The aim of this investigation was to discover antibodies against blood clotting factors by the passive hemagglutination test (PHT) and enzyme-like immunoadsorption assay (ELISA).

EXPERIMENTAL METHOD

Human, bovine, canine, porcine, and murine factor Xa (Stuart-Prower factor) was obtained from activated human and animal prothrombin complex by iron-exchange chromatography on DEAE-Sephadex A-50 (Pharmacia, Sweden) [1]. Bovine thrombin (Research Institute of Epidemiology, Kaunas) and human thrombin (Leningrad Institute of Hematology and Blood Transfusion) were additionally purified by gel-filtration on Sephadex G-100 (Pharmacia) [2]. Canine thrombin was obtained from prothrombin by the method described in [7]. All preparations were freeze-dried and stored at 4°C.

For the PHT, thrice washed sheep's erythrocytes were sensitized with a 0.25% solution of glutaraldehyde (Merck, West Germany) and with factors Xa and IIa of different origin. The erythrocytic diagnostic preparations were used in the form of a 2.5% suspension in buffered physiological saline (BPS) and kept at 4°C for not more than 1 week. The effectiveness of sensitization was monitored on the basis of reactions with specific antisera.

The following materials were used for ELISA. The IgG fraction was isolated from sheep antiserum against human IgG (N. F. Gamelya Institute of Epidemiology and Microbiology) by salting out twice with ammonium sulfate and by ion-exchange chromatography on DEAE-Sephadex. After concentration to 10 mg/ml with the aid of PEG-6000 (from Loba-Chemie, Austria) the im-

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TABLE 1. Content of Antibodies in Plasma and Blood Serum of Man and Animals against Autologous Enzymes (M ± m; $\log_2$)

Test object	Factor IIa	Factor Xa
Human plasma	6,0±0,3	3,3±0,3
Human serum	0	0
Pig plasma	—	3,5±0,4
Pig serum		0
Bovine plasma	6,0±0,2	4,0±0,3
Bovine serum	0	0
Dog plasma	4,8±0,7	3,3±0,5
Dog serum	0	0
Rat plasma	—	3,5±0,3
Rat serum	—	0

TABLE 2. Autoantibodies against Blood Clotting Factors Detected by ELISA in Human Plasma (in extinction units)

Dilution of plasma	Factor IIa	Factor Xa
1/2	0,029±0,0013	0,022±0,0025
1/256	0,004±0,0004	0,015±0,0016

munoglobulins were conjugated by sodium periodate (from Serva, West Germany) with horseradish peroxidase (from Reanal, Hungary), purified before hand to $R_Z \geqslant 3$. The conjugation procedure was carried out as described previously [11]. The resulting conjugate was subjected to gel-filtration on Sephadex G-200 (Pharmacia). The fractions of the first peak were pooled, concentrated to a volume of 2 ml, and tested for ability to interact with antigen (human IgG) in the precipitation test in agar (Difco, USA) and the PHT with erythrocytes sensitized by the human gamma-fraction (N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR). Enzyme activity of the conjugate was estimated by interaction with a substrate (3,3-dianisidine, from Serva). ELISA was carried out as follows. Wells in plates (Dynateck, USA) were sensitized with 5 μg of human factors Xa or IIa. Citrated donor's plasma (a source of detectable autoantibodies against blood clotting factors) was then added. After incubation for 1 h at 37°C the wells were washed three times with BPS containing 0.05% Tween-20 solution, the conjugate was added in a working dilution of 1:40, and incubated, after which it was washed and the substrate was added. The reaction was stopped by addition of a 3% solution of sodium azide (from Serva). The intensity of the reaction was assessed on an MR 580 automatic analyzer (Dynateck), using a dual-beam system (λ = 490-450 nm) and expressed in extinction units. The numerical results were subjected to statistical analysis [6].

EXPERIMENTAL RESULTS

In the writers' view human and animal plasma contain autoantibodies capable of inhibiting specific enzyme activity. In fact, antibodies against blood clotting factors of autologous origin (Table 1) were regularly recorded in the PHT with plasma obtained from healthy blood donors and intact animals (Table 1). Meanwhile, virtually no autoantibodies were discovered in the serum. The explanation of this finding is as follows. During fibrin formation active procoagulants are generated, including factors Xa and IIa. The latter bind autoantibodies present, so that they cannot be found in the serum. For instance, it was shown previously that autoantibodies are detectable in extremely low titers [9] in the gamma-fraction isolated from the serum. Similar results were reported by Friedman [10], who showed that antoantibodies against thrombin disappear in serum, although they are found in plasma.

All the tested samples of plasma gave positive results in ELISA (Table 2). The high sensitivity of the method will be noted. Autoantibodies against factors IIa and Xa were recorded in plasma diluted 256 times. However, it will be recalled that this procedure used to determine autoantibodies in plasma against blood clotting factors gives definitely underestimated results, for both autoantibodies and inhibitors of serine proteases (antithrombin III, α_2-macroglobulin, α_1-antitrypsin, and so on) compete for interaction with factors IIa and Xa, immobilized on wells in the plates. The same applies also to the results of the PHT. The above-mentioned inhibitors evidently mask to some degree the autoantigenic determinants of active procoagulants. It is not surprising, therefore, that the titer of autoantibodies in the PHT is much higher in the gamma-fraction isolated from the test plasma [8].

The results indicate the existence of a physiological immune mechanism for the regulation of enzyme homeostasis in the blood clotting system.

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