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DETERMINATION OF SERUM ANTIPLATELET ANTIBODIES IN PATIENTS WITH IDIOPATHIC THROMBOCYTOPENIC PURPURA BY ELISA

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Improvements in methods of recording antiplatelet antibodies (AB) in patients with idiopathic thrombocytopenic purpura (ITP) have led to improvements in the diagnosis and evaluation of the effectiveness of treatment of this disease. Direct and indirect methods of determination of antiplatelet AB are distinguished. In the first case the quantity of antibodies associated with platelets is determined, in the second case the quantity of antiplatelet AB in the patient's serum. Various methods have been suggested for recording antiplatelet AB. Agglutination tests are nowadays considered to be inefficient. These tests do not reveal incomplete AB and they are attended by the difficulty of setting up a precise negative control, due to the ability of platelets to aggregate [1]. Dixon and co-workers [3] were among the first to develop a method of quantitative determination of antiplatelet AB based on inhibition of complement-dependent lysis of erythrocytes, correlating with the use of antiglobulin AB. Immunofluorescence [8] and radioimmune methods [2, 5] of recording AB associated with platelets and circulating AB also have been successfully used. However, immunoenzyme methods and, in particular, enzyme-linked immunosorbent assay (ELISA) are most widely used at the present time [3, 7]. Advantages of ELISA include the possibility of simultaneous determination of AB in several samples, quantitative estimation of AB, relative simplicity, and sufficiently high reproducibility.

This paper describes an indirect method of ELISA for recording serum antiplatelet AB on the basis of their binding with healthy human platelets immobilized on plastic.

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EXPERIMENTAL METHOD

The following groups of patients were studied: 10 patients with ITP during a flare-up of the disease (platelet count below 30,000/μl), three of whom had received many blood transfusions; one with systemic lupus erythematosus (SLE), one with partial red cell aplasia (PRCA), who had received many blood transfusions. The control group consisted of nine healthy group AB (IV) blood donors. Sera of the patients and healthy blood donors were kept at −20°C and were thawed not more than once or twice.

The sera were tested on platelets from healthy group 0 (I) blood donors. The platelets were washed to remove plasma by the method in [6] with modifications. Blood was collected in acid citrate-dextrose in the ratio of blood:anticoagulant (6:1). Platelet-enriched plasma was obtained by centrifugation at 180 g for 15 min at room temperature. The platelets were then sedimented at 1000g for 15 min at room temperature and washed twice in Tyrode solution with Na citrate: 137 mM NaCl, 2.7 mM KCl, 0.36 mM NaH_2PO_4, 30 mM Na citrate, 1 mM $MgCl_2$, 0.1% dextrose solution, 0.35% bovine serum albumin solution (BSA, from "Sigma"), 0.2 U/ml of apyrase (ADPase activity, from "Sigma"), and 10 mg/ml of prostaglandin E_1 (Kaunas Medical Reagents Factory), pH 6.5. The first washings also were treated with 25 U/ml of heparin ("Sigma"). After the last washing the platelets were resuspended in Tyrode solution of the following composition: 137 mM NaCl, 2.7 mM KCl, 0.36 mM NaH_2PO_4, 5 mM HEPES, 1 mM $MgCl_2$, 0.1% dextrose solution, and 0.35% BSA solution, pH 7.4. After washing, the platelets were counted in an automatic PL100 Platelet Counter (TOA Medical Electronics, Japan) and adjusted with Tyrode solution to a count of $2 \cdot 10^8$/ml.

The platelets were introduced in a volume of 100 μl into wells of a 96-well microtest panel ("Nunc," Denmark) and sedimented on the bottom of the wells in a "Beckman TJ-6" centrifuge (USA) at 1000g for 5 min. To obtain better adhesion the platelets were incubated after sedimentation for 15 min at 37°C, after which the nonadherent platelets were washed off with Tyrode solution. The plastic was blocked by incubating the wells with platelets and control wells without platelets with 1% BSA solution in Tyrode solution for 40 min at 37°C. Next, 50 μl of the sera in titers of 3-2189 was added to each well with platelets and to each control well, by diluting them successively 1:3 with Tyrode solution containing 1% BSA solution. Incubation with the sera continued for 40 min at 37°C, after which the wells were washed at least 5 times with Tyrode solution. After washing, 50 μl sheep's AB against human immunoglobulins, conjugated with peroxidase (the AB was supplied by S. P. Domogatskii, Laboratory of Immunomorphology, Institute of Experimental Cardiology, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR) was added to each well. The AB concentration was chosen beforehand on the basis of binding with human immunoglobulins, immobilized on plastic. Incubation with the second AB continued for 30 min at room temperature and unbound AB were washed off at least 5 times, and the chromogenic substrate (10 μg/ml of orthophenylenediamine, 0.005% hydrogen peroxide solution in 10 mM citrate buffer, pH 4.5) was added to the wells. The reaction was stopped after 3-5 min by the addition of a 50% solution of sulfuric acid and development of the color was recorded by measuring absorption at 492 nm (A_{492}) in a "Titertek Multiscan MCC" spectrophotometer (Finland). During analysis of the data, values obtained in the control wells (nonspecific sorption) were first subtracted from values of A_{492} recorded in the wells with platelets. In each experiment, serum from a patient with PRCA was used as the positive control, and one of the sera from healthy group AB (IV) blood donors as the negative control. Responses for which the values of A_{492} exceeded the average A_{492} for the negative controls by an amount equal to two standard deviations (2σ) were considered to be positive.

EXPERIMENTAL RESULTS

Serum AB bound with platelets were found in five patients with ITP, one with PRCA, and one with SLE (Figs. 1 and 2). The highest AB titers were found in the patient with PRCA (Fig. 1). The serum from this patient, used as the positive control, and sera from healthy group AB (IV) blood donors, used as the negative controls, were tested on platelets from many donors. Values of A_{492} for the positive control varies between 0.566 and 0.277 (mean ±σ 0.400 ± 0.107; n = 9), whereas for the negative controls, they varied between 0.163 and 0.02 (mean ±σ 0.065 ± 0.048, n = 9), evidence of the sufficiently high reproducibility of the results in different experiments.

The presence of serum antiplatelet AB in the patient with PRCA and three patients with ITP, who had received multiple blood transfusions, was evidently due to the presence of isoimmune AB in the blood. The discovery of antiplatelet AB in sera of two other patients with

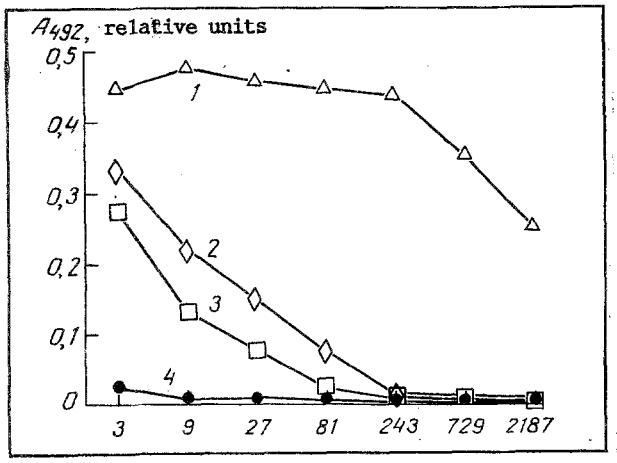

Fig. 1

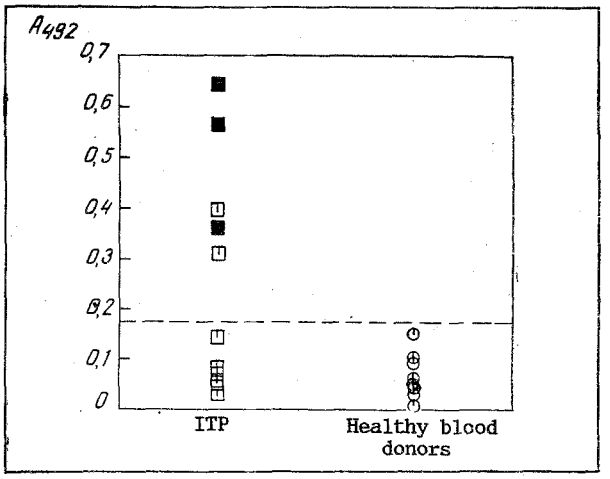

Fig. 2

Fig. 1. Binding of AB from sera of patient with PRCA with multiple blood transfusions (1), patient with ITP in a flare-up stage (2), a patient with SLE (3), and a healthy group 0 (I) blood donor with blood group AB (IV) platelets immobilized on plastic. Sera were titrated by diluting them serially with Tyrode solution in 1% BSA solution. Abscissa, dilution of serum.

Fig. 2. Sera of 10 patients with ITP and nine healthy blood donors were tested on platelets of blood group AB (IV) donors, immobilized on plastic. Unshaded squares – patients with ITP, black squares – patients with ITP after multiple blood transfusions, empty circles – healthy blood donors. Responses differing from the mean value of the negative control (0.065) by more than twice the standard deviation (2σ = 0.096) were considered positive. Threshold value indicated by a broken line.

ITP (a man and a childless woman) and of a childless woman with SLE, who had not received blood transfusions, confirms the autoimmune nature of the disease in these patients. No serum AB, bound with platelets from healthy blood donors were found in five patients with ITP. However, this does not rule out the possibility of detection of allo-AB or auto-AB associated with patients' platelets by the use of a direct method.

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