DETERMINATION OF ANTIPLATELET ANTIBODIES ON THE SURFACE OF PLATELETS FROM PATIENTS WITH VARIOUS FORMS OF IMMUNE THROMBOCYTOPENIA BY DIRECT RADIOIMMUNOASSAY

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Antiplatelet antibodies (APA) play an important role in the pathogenesis of idiopathic thrombocytopenic purpura (ATP) and other forms of immune thrombocytopenia [1, 8, 10, 11]. Methods of radioimmunoassay and enzyme immunoassay (RIA and EIA respectively), Dixon's method, the immunofluorescence method, and also methods based on the use of the ^{125}I-labeled C3 fragment of complement and ^{125}I-labeled staphylococcal protein A have been developed in order to study the APA level [2-4, 10, 15-17, 19]. These methods have been used to determine APA both in serum (indirect method) and on the surface of platelets (direct method). The writers previously developed a method of indirect EIA to study APA in the blood serum of patients with various types of immune thrombocytopenia [2]. By this method APA were discovered in the blood serum of patients with ITP, systemic lupus erythematosus (SLE), and patients undergoing multiple blood transfusions. However, serum APA were not found in all patients with immune thrombocytopenias.

In some cases APA may be absent from the serum but present on the surface of the platelets [4, 6, 11]. In these cases APA can usually be found on the basis of a raised level of platelet-associated immunoglobulins, i.e., by the direct method.

In this paper we suggest a method of direct RIA based on the use of ^{125}I-labeled antibodies (^{125}I-AB), directed against human immunoglobulins.

EXPERIMENTAL METHOD

By the method of direct RIA AB were detected on the surface of the platelets of the following groups of patients: in 12 patients with ITP and in 27 patients with chronic lymphatic leukemia (CLL), complicated by the development of thrombocytopenia, with a platelet count of under 100,000/μl. The serum APA content was determined by the method of indirect EIA as described previously [2] in 17 patients with ITP and 54 patients with CLL, with a platelet count of under 100,000/μl blood serum. The control group comprised six healthy blood donors. All patients with ITP were on steroid therapy. In three patients with CLL, AB associated with the platelet membrane was studied before and after courses of steroid and cytostatic therapy. The APA level on the surface of the platelets was studied by RIA, using sheep AB directed against human IgG. The AB were generously provided by G. L. Idel'son, on the staff of the All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR. The AB were labeled with ^{125}I by the iodogen method [7]. Iodogen ("Pierce," USA) was dissolved in chloroform in a concentration of 20 μg/ml, the volume made up to 300 μl in glass test tubes, after which the chloroform was removed by evaporation in vacuo. For labeling 0.5 mg of AB in 70 μl phosphate-salt buffer (PSB), pH 7.4, was introduced into a test tube and treated with iodogen, 0.5 mCi of Na ^{125}I ("Reakhim," USSR) was added, and the sample was incubated, with mixing, for 5 min at room temperature. The labeled AB were separated from

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TABLE 1. Binding of ^{125}I-AB Against Human Immunoglobulins with Healthy Human Immunoglobulins with Healthy Human Platelets, Treated with Sera of a Healthy Individual and of Patients with ITP and CLL, Complicated by Thrombocytopenia

Platelets	Binding of ^{125}I-AB against human immunoglobulins, ng/10^6 platelets
Platelets treated with sera of	
healthy individual	0.50
patient with ITP	4.43
patient with CLL	1.30

Legend. Healthy human platelets, washed to remove plasma, were incubated with sera of a healthy individual and of patients with ITP and CLL. After incubation, immunoglobulins associated with platelets were determined on the basis of binding of ^{125}I-AB.

free ^{125}I by chromatography through Sephadex G-25 on a PD-10 column ("Pharmacia," Sweden). The specific activity of the AB was usually from 1000 to 1500 cpm/ng protein. The working concentration of ^{125}I-AB was chosen beforehand on the basis of their binding with human IgG, immobilized on plastic.

Blood was taken into a 5% solution of EDTA, pH 7.4, as the anticoagulant, with a ratio of 1:9 to the volume of blood withdrawn. Platelet-enriched plasma was obtained by centrifugation for 15 min at 150 g. Next, by centrifuging the platelet-enriched plasma for 20 min at 1000 g the residue of platelets was separated. The platelets were washed 3 times to remove plasma with acid PSB, pH 6.5, containing 5 mM EDTA, by centrifugation at 1000 g for 20 min at room temperature. After the last sedimentation the platelets were suspended in 500 μl of PSB, pH 7.4, containing 5 mM EDTA and 4% bovine serum albumin (BSA, from "Serva," Austria). The platelet concentration was determined with the aid of a cell counter (PL-100, from "Toya Medical Electronics Systems," Japan) and adjusted to $4 \cdot 10^8$/ml. Next, ^{125}I-AB against human IgG were added to the platelets in a concentration of 10 μg/ml and the sample was incubated at 37°C for 30 min. Unbound ^{125}I-AB was separated from the platelets by centrifugation through 20% sucrose. For this purpose 100 μl of incubation mixture was layered above 400 μl of 20% sucrose in PSB, pH 7.4, 5 mM EDTA, 2% BSA in 500 μl plastic test tubes ("Eppendorf," Germany) and centrifuged for 5 min at 9800 g in an "Eppendorf-5414" centrifuge (Germany). The tubes were frozen, after which the base of the tube containing the residue of platelets was cut off and the upper and lower parts of the tube were counted separately, so that radioactivity bound with the cells and free radioactivity could be determined. In the control samples ^{125}I-AB were added to PSB without platelets and the tests were carried out in the same way as those with platelets. Usually the background values of the test without platelets did not exceed 1000 cpm, or under 0.5% of the introduced radioactivity. The level of binding of the ^{125}I-AB with platelets was estimated in nanograms ^{125}I-AB/10^6 platelets and (or) as a percentage of the introduced radioactivity. Each blood sample was tested in two parallel determinations. Responses exceeding the mean level in the control donors by twice the standard deviation were considered to be positive (mean + 2σ = 0.354 + 0.24 ng/10^6 platelets). In preliminary experiments the ^{125}I-AB used also were tested in experiments to determine serum AB by the indirect RIA method. For this purpose platelets from a healthy donor were used and treated with serum from patients with ITP and with CLL and thrombocytopenia, and from the healthy donor. The patient's sera contained APA, which had previously been revealed by the indirect EIA method [2]. After incubation with sera the platelets were washed 3 times with PSB containing 5 mM EDTA and 45 mM BSA, after which the bound serum AB were determined by incubating the platelets with ^{125}I-AB against human immunoglobulins. The tests were carried out in the same way as was described above for the direct method. The results were subjected to statistical analysis on a "Labtam-3003" computer, using the "Statpack" program (USA).

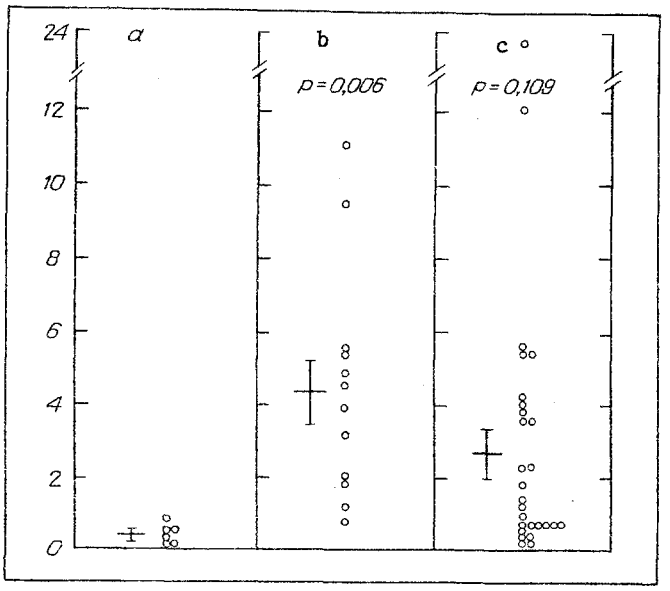

Fig. 1. Determination of immunoglobulins associated with the platelet surface in healthy individuals (a) and patients with ITP (b) and with CLL, containing fewer than 100,000 platelets/μl. Content of surface immunoglobulins was determined by binding of ^{125}I-AB against human immunoglobulins with platelets. Individual values and mean ± error of the means are given. Significance of differences (p) between groups of patients and healthy individuals calculated by Student's t test for means. Ordinate, binding of ^{125}I-AB (in ng/10^6 platelets).

EXPERIMENTAL RESULTS

In preliminary experiments ^{125}I-AB against human immunoglobulins were tested by indirect RIA. In these experiments the sera used had previously been tested by indirect EIA [2] and, according to the results obtained by that method, they contained APA. After incubation of platelets with these sera, they bound several times more ^{125}I-AB against human immunoglobulins than platelets incubated with the sera of the healthy donor (Table 1). It was concluded from these data that the ^{125}I-AB which we used can also be used in the direct method, i.e., for determination of AB directly on the surface of platelets of patients with thrombocytopenias.

Results of determination of AB associated with the platelet surface in the direct method are shown in Fig. 1. Among 12 patients with ITP the control level of platelet-associated immunoglobulins (mean + 2σ = 0.354 + 0.24 ng/10^6 platelets) exceeded the control level in all 12 cases. The mean concentration of immunoglobulins on the surface of the platelets in patients with ITP was about 7 times higher than in healthy individuals ($p = 0.006$). In the group of patients with CLL complicated by thrombocytopenia below 100,000/μl an increase in the level of immunoglobulins associated with the platelet surface was found in 21 of the 27 subjects tested, and in six patients the immunoglobulin concentration did not exceed the control level. Despite the very high values for the concentration of platelet-associated immunoglobulins in some patients with CLL (10 times or more higher than the control level), the presence of a subgroup with a normal immunoglobulin level prevented the discovery of significant differences between the mean values in the groups of patients and normal individuals ($p = 0.109$). Evidently in patients with CLL, thrombocytopenia does not develop only by a mechanism due to the presence of APA. In particular, besides this mechanism, depression of the megakaryocytic branch of the bone marrow by leukemic lymphocytes may also take place [5].

Comparison of the results obtained by RIA and the indirect EIA methods confirms data in the literature [4, 6] according to which the frequency of detection of AB on the surface of platelets is much higher than the frequency of detection of circulating serum AB. For instance, the percentage of positive "responses" in the group of patients with ITP, when the method of direct RIA was used, was 100% (all 12 of the 12 patients tested), but when the indirect EIA method

TABLE 2. Effect of Steroid and Cytostatic Therapy on Platelet Count and Level of Platelet-Associated Immunoglobulins in Patients with CLL, Complicated by Thrombocytopenia

Patient	Number of platelets in 1 μl		Binding of ^{125}I-AB with platelets, ng/10^6	
	before treatment	after treatment	before treatment	after treatment
K.	39 000	190 000	5,25	0,20
B.	60 000	268 000	5,00	0,30
S.	93 000	168 000	1,80	0,45

Legend. Patients with CLL complicated by thrombocytopenia were treated with steroid hormones or cytostatics. Platelet count and level of APA on surface of platelets were determined before beginning and after end of treatment.

was used, it was only 35.2% (six of 17), and in the group of patients with CLL (with a platelet count of below $10^6/\mu l$) the corresponding values were 77.8% (21 of 27) and 13.0% (seven of 54).

Table 2 gives the results of investigation of APA on the surface of platelets and of the platelet count depending on treatment given. Table 2 shows that among patients with CLL complicated by thrombocytopenia and responding to treatment by steroid drugs and cytostatics, an increase in the platelet count was accompanied by a fall of the AB level on the platelet surface.

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