METHODS

Assay of Antigens of Antiplatelet Antibodies in Patients with Various Thrombocytopenias

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Sandwich enzyme-linked immunosorbent assay was developed for evaluation of target antigens reacting with antiplatelet auto- and alloantibodies. The method based on specific immobilization of platelet antigens via monoclonal antibodies was applied to identify antigens of platelet-associated and serum autoantibodies in patients with different thrombocytopenias and to determine platelet HPA-1 alloantigens.

Key Words: *thrombocytopenia; antiplatelet antibodies; platelet glycoproteins; enzyme-linked immunosorbent assay*

Immune thrombocytopenias are the most common cause of reduced blood platelet count. Immune thrombocytopenic purpura (ITP) is caused by autoantibodies against autogenous platelet antigens, either normal or altered by drugs, viruses, or other agents. Neonatal and posttransfusion thrombocytopenis are characterized by antibody production against fetal or donor platelet alloantigens, respectively. These antibodies interact with specific human platelet alloantigens (HPA antigens) or major histocompatibility complex antigens (HLA antigens). In posttransfusion thrombocytopenia, alloantibodies primarily induce rapid destruction of transfused platelets and then interact with autogenous platelets. Neonatal thrombocytopenia is caused by feto-maternal incompatibility by some platelet antigen which is expressed on paternal and fetal platelets but is absent in maternal cells. This results in enhanced platelet destruction and thrombocytopenia in the fetus and neonate [2,5,6,11].

Apart from detection of platelet-associated immunoglobulins (PA-IgG) and platelet-reactive serum antibodies, various thrombocytopenias are now diagnosed by methods based on identification of platelet auto- and alloantigens. Identification of target molecules for antiplatelet antibodies makes it possible to differentiate production of specific antiplatelet antibodies and nonspecific elevation of PA-IgG, as well as to distinguish antibodies against platelet-specific antigens from anti-HLA antibodies in neonatal and posttransfusion thrombocytopenia [6,7,9].

Here we describe a procedure of sandwich enzyme-linked immunoassay allowing for identification of target-antigens of PA- and serum antiplatelet antibodies. The method based on monoclonal antibody-specific immobilization of platelet antigens (MAIPA) [6,7,9] was applied to identify autoantigens in patients with different thrombocytopenis and to determine platelet HPA-1 alloantigens.

MATERIALS AND METHODS

A total of 7 patients with ITP and 2 patients with lymphoproliferative diseases (LPD) were examined. In all patients platelet count was below 30×10^9 cells/liter. Control group comprised healthy donors with a platelet count above 200×10^9 cells/liter. PA-IgG was assayed by radioimmunoassay [4], and the content of serum platelet-reactive antibodies was measured by enzyme-linked immunosorbent assay [3].

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Antigens for antiplatelet antibodies were determined using sandwich enzyme-linked assay based on (MAIPA) [6,7,9]. The following monoclonal antibodies against the major platelet glycoproteins (GP) were used: AP3 against GP IIIa (kindly supplied by Dr. P. J. Newman, Blood Center of South-Eastern Wisconsin, USA), VM16a against GP IIb-IIIa complex, VM16d against GP Ib (produced by us [1,9]). Monoclonal antibodies against Tag-polymerase (kindly provided by Dr. T. N. Vlasik, Russian Research and Production Center), which do not react with platelets, were used as negative control. Antisera against GP IIIa — HPA-1a and 1b (PLA1 and 2 according to previous nomenclature) were kindly supplied by Dr. J. McFarland (Blood Center of South-Eastern Wisconsin, USA).

Platelets isolated from peripheral blood of patients and donors [1] were resuspended in phosphate buffered saline (PBC), adjusted to a concentration of 10^8 cells/ml, and lysed on ice in the presence of phenylmethylsulfonyl fluoride (0.5 mM) and 1% Triton X-100 for 30 min. Insoluble material was precipitated for 5 min at 10,000*g*. The lysate was used immediately or stored at -70°C. Monoclonal antibodies were adjusted with 200 mM carbonate buffered saline (pH 9.6) to a concentration of 10 μg/ml and incubated in microtitration plates (Nunc, 50 μl) at 4°C for at least 12 h. The plates were then washed with PBS containing 0.05% Tween-20 (PBS/Tween), and nonspecific binding sites were blocked with 1% gelatin (1 h, room temperature). Platelet lysate from patient (direct MAIPA) or donor (indirect MAIPA) was transferred to plates 50 μl) and incubated for 1 h at room temperature. After the incubation, the plates were twice washed with PBS/Tween with 1% Triton X-100 and 5 times with PBS/Tween. For indirect MAIPA, incubation with platelet lysate and subsequent washout were followed by 1 h incubation of the same plate with patient serum diluted 4-fold with 1% gelatin in PBS and 5-fold washout with PBS/Tween. To assess antibody binding to immobilized antigens, 50 μl peroxidase-labeled anti-human IgG antibodies (BioRad) in 1% gelatin (1:2000 dilution) were added to each well, incubated for 30 min at room temperature, washed, and antibody binding was recorded using a chromogenic substrate (o-phenyldiamine) by measuring light absorbance at 492 nm. In each experiment, antiserum against GP IIIa alloantigen — HPA-1a (PLA1) and serum of healthy donor were used as positive and negative controls, respectively. For each serum, three independent experiments with different platelet preparations were performed. For each lysate (direct MAIPA) and each serum (indirect MAIPA) nonspecific binding (A_{492} in wells with control antibodies

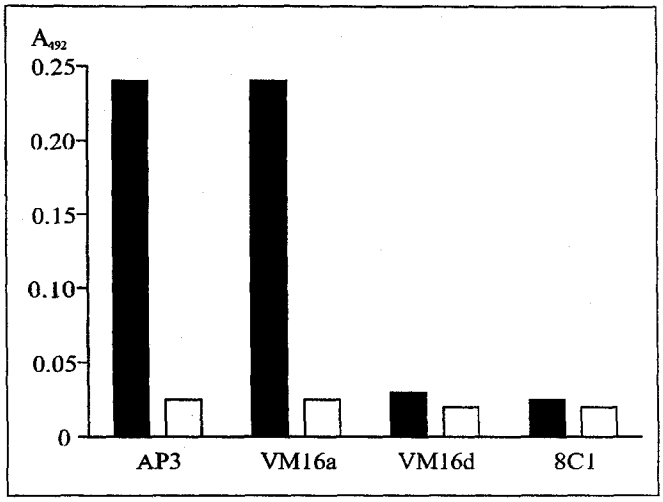

Fig. 1. Binding of platelet-associated antibodies from patient with thrombocytopenic purpura (ITP, Table 1, patient 1) with monoclonal antibody immobilized platelet antigens (direct MAIPA assay). Antigens were immobilized on antibodies against glycoproteins IIb-IIIa complex (AP3 and VM16a antibodies) and Ib (VM16d antibodies); 8C1 antibodies which do not interact with platelet antigens were used as negative control. Platelet lysate from ITP patient (dark bars) or healthy donor (open bars) was added to immobilized antigens and antigen-bound human immunoglobulins were evaluated by enzyme-linked immunosorbent assay.

8C1) was subtracted from experimental A_{492} values (wells with specific antibodies against platelet GP) and the resultant values were compared with those of healthy donors (negative control). The test was regarded positive when A_{492} for patients more than 2-fold surpassed that for healthy donors.

RESULTS

Specificity of autoantibodies was evaluated in patients with low platelet count and high levels of PA-IgG and/or serum antibodies interacting with normal human platelets.

Monoclonal antibodies against GP-IIb-IIIa complex and GP IIb, the major surface platelet antigens, and control monoclonal antibodies which do not interact with platelets adsorbed to plastic multiwell plates, were incubated with platelet lysate from patients (PA-IgG assay, direct MAIPA) or from healthy donors and then with patient serum (measurement of antigens for serum antibodies, indirect MAIPA). Autoantibodies associated with monoclonal antibody-immobilized antigens were assessed using peroxidase-labeled anti-human IgG antibodies.

Figure 1 shows typical results of direct MAIPA-assay of platelet lysate from Patient 1 with ITP and high level of PA-IgG (Table 1). An intensive binding of anti-human IgG antibodies is seen in wells containing GP IIb-IIIa complex from patient with ITP immobilized by monoclonal MV16a and AP3 anti-

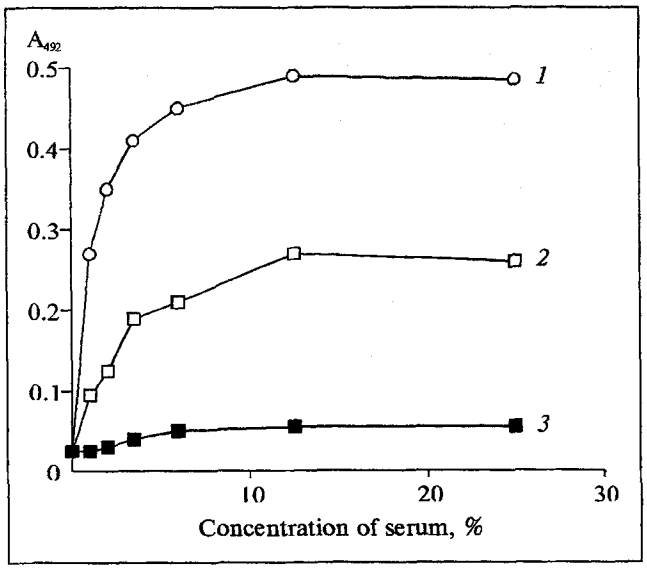

Fig. 2. Binding of serum anti-HPA-1a (anti-PLA) antibodies and serum antibodies from patient with thrombocytopenic purpura (ITP) to monoclonal antibody immobilized platelet glycoprotein IIb-IIIa complex (indirect MAIPA assay). Monoclonal antibodies against glycoprotein IIb-IIIa complex (VM16a) adsorbed on plastic were incubated with lysate of donor platelets and then with varied concentrations of anti-HPA-1a serum (*1*), serum of IPT patient (Table 1, patient 1) (*2*), or healthy donor (*3*) were added. Binding of human immunoglobulins to immobilized glycoprotein IIb-IIIa was measured by enzyme-linked immunosorbent assay. A_{492} in wells containing control antibodies 8C1 (do not interact with platelet antigens, nonspecific binding) did not exceeded 0.1.

bodies. A low binding is observed in wells containing GP IIb-IIIa complex from healthy donor, GP Ib immobilized by VM16d, and control 8C1 antibodies. These data suggest that PA-IgG in this patient interact with GP IIb-IIIa complex.

Figure 2 shows the data of indirect MAIPA analysis of the following sera: antiserum against HPA-1a (PLA1), the main platelet alloantigen localized in GP IIIa [10,11], serum from ITP patient with high level of antiplatelet antibodies (Table 1, Patient 1), and serum from healthy donor. Various concentrations of the sera were added to immobilized GP IIb-IIIa (VM16a monoclonal antibodies) from normal platelet lysate. Both anti-HPA-1a and serum from IPT patient intensively bind to immobilized GP IIb-IIIa.

The contents of PA-IgG and serum antiplatelet antibodies and their target antigens in patients with ITP (n=7) and LPD (n=2) are enlisted in Table 1. The content of PA-IgG substantially increased in ITP patients and remained virtually unchanged or slightly increased in LPD patients. Serum antibodies were found in 5 patients with IPT and in 2 LPD patients. Specificity of PA-IgG was assayed by direct MAIPA assay. Four patients with ITP (Nos. 1-4) had PA-IgG against GP IIb-IIIa and patient 3 had also anti-GP Ib antibodies. PA-IgG were not assayed in 3 patients with IPT (Nos. 5-7) because of low platelet content in the samples and in 2 patients with LPD because of low PA-IgG content. It should be noted that in 2 patients (Nos. 3 and 4) no serum antibodies interacting with normal platelets were found despite the presence of antiplatelet Pa-IgG. It can be hypothesized that antiplatelet autoantibodies in these patients (they were children) are directed toward altered platelet antigens and therefore do not interact with native antigens on normal platelets. Antigen specificity of serum antibodies was assessed in indirect MAIPA test. Two patients with ITP (Nos.

TABLE 1. Antiplatelet Antibodies and Target Antigens in Patients with ITP and LPD

No.	Diagnosis	PA-IgG	Serum antibodies	Antibodies against			
				GP IIb-IIIa		GP Ib	
				PA-IgG	serum antibodies	PA-IgG	serum antibodies
1	ИТП	1100	420	440	350	100	90
2	ИТП	2200	550	1300	120	100	110
3	ИТП	850	110	840	n.d.	550	n.d.
4	ИТП	440	100	500	n.d.	100	n.d.
5	ИТП	950	500	n.d.	100	n.d.	90
6	ИТП	630	300	n.d.	140	n.d.	160
7	ИТП	1200	630	n.d.	590	n.d.	130
8	ЛПЗ	100	900	n.d.	130	n.d.	140
9	ЛПЗ	210	500	н.о.	100	н.о.	120

Note. The values are percentage of control (healthy donors, 100%); n.d., not determined.

1 and 7) had serum antigens against GP IIb-IIIa. In 5 patients (3 patients with ITP and 2 with LPD) no antibody binding to immobilized GP IIb-IIIa and Ib antigens were detected despite the high level of serum platelet-reactive antibodies. The following explanations can be proposed. First, these patients probably had anti-HLA but not specific antiplatelet antibodies. This is most likely for patients Nos. 2,5,7,8, who underwent repeated platelet transfusion. The presence of anti-HLA antibodies in patients with LPD (Nos. 8 and 9) explains unchanged content of PA-IgG in combination with level of serum antibodies interacting donor platelets. Another explanation is that serum antibodies interact with conformation-liable epitopes whose antigenic structure are affected by detergent treatment during lysis procedure preceding antigen immobilization.

Neonatal thrombocytopenia usually results from parental incompatibility by HPA-1 (PLA) alloantigen [2,5,11] existing in two alleles HPA-1a and 1b (PLA1 and 2) and differing by substitution of leucine-33 to proline in GP IIIa molecule [10,11].

In the next experimental series we determined antigen HPA-1 phenotype in 26 donors (Moscow strain) by indirect MAIPA test using patient sera containing anti-HPA-1a and 1b antibodies. We found that 18 donors had HPA-1a/1a phenotype (69%), 7 persons had HPA-1a/1b phenotype (27%) and 1 donor had HPA-1b/1b phenotype, which is consistent with the alloantigen frequency in other European countries [10,11].

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