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FLOW CYTOMETRIC ANALYSIS OF ANTI-PLATELET ANTIBODIES IN IDIOPATHIC THROMBOCYTOPENIC PURPURA

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ABSTRACT

Anti-platelet antibody measurement may be important in defining the pathogenesis of thrombocytopenic states. In this paper we compared three anti-human immunoglobulin reagents by using them to detect anti-platelet antibodies on the platelet surface and in the serum of 14 patients with chronic idiopathic thrombocytopenic purpura (ITP) and 22 thrombocytopenic disorders. Samples were analyzed by both flow cytometry and a fluorescence microscope. In ITP patients, the direct test was positive in 50% of the cases, while the indirect technique proved to be positive in a slighty higher number of those tested (56%). Furthermore, the number of positive cases was similar for the three reagents used in this study, although the mean percentage of positive platelets was higher for the kappa/lambda monoclonal reagent. These data futher support the sensitivity and reproducibility of flow cytometry analysis, which was capable of detecting anti-platelet antibodies in all patients with transfused Cooley's disease (regarded as positive control), as well as in a significant number of patients with ITP or related diseases. On the basis of the data presented here, definitive proof regarding the presence of anti-platelet antibodies in patients with thrombocytopenia still has to be found, and further studies are needed in order to ascertain the autoimmune nature of these disorders.

Key words: anti-platelet antibodies, ITP, flow cytometry, anti-Ig immunoglobulins

Anti-platelet antibody measurement provides useful information on the causes of thrombocytopenic diseases. In the last few years flow cytometry analysis has been increasingly used to detect anti-platelet antibodies either in the serum or on the surface of platelets from patients with chronic idiopathic thrombocytopenic purpura (ITP) or other thrombocytopenic disorders.[1]

In this paper we compared three anti-human immunoglobulin reagents (B-Cell SIg marker-Ortho; total anti-human immunoglobulins FITC-Ylem; rabbit anti-human k light chains/FITC and rabbit anti-human λ light chains/RPE-Dako) by using them to detect anti-platelet antibodies in the serum and on the surface of platelets obtained from 14 patients with chronic ITP and 22 thrombocytopenic states associated or not associated with hematologic malignancies (15 chronic lymphoproliferative disorders, 5 liver diseases, 2 systemic lupus erythematosus). Specimens were analyzed by both flow cytometry (FCM) and a standard fluorescence microscope (LM).[2]

Materials and methods

Platelet suspensions from EDTA-anticoagulated blood were washed three times with 3 mL of phosphate buffer solution/bovine serum albumin (PBS/BSA) 0.1%, resuspended in 2 mL of paraformaldehyde at a dilution of 1% to prevent non-immune binding, and washed twice more with PBS.[3]

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Table 1. Comparative evaluation of serum anti-platelet antibody measurement by flow cytometry and light microscope (indirect technique).

	F C M			L M		
Subjects	+	±	–	+	±	–
AB+ donors	/	/	0/36 (0%)	/	/	0/36 (0%)
positive controls	36/36 (100%)	/	/	36/36 (100%)	/	/
ITP	5/14 (35%)	3/14 (21%)	6/14 (43%)	4/14 (28%)	4/14 (28%)	6/14 (43%)
thrombocytop. states	6/22 (27%)	4/22 (18%)	12/22 (54%)	7/22 (32%)	1/22 (4%)	14/22 (64%)

The results are expressed as number of positive cases and mean percentage of positivity (in parentheses)
Legend: FCM=flow cytometry; LM=light microscope; ITP=idiopathic thrombocytopenic purpura
+: strong positivity (> 20% of the platelets ananalyzed)
±: weak positivity (15-20% of the platelets analyzed)
–: negativity (< 15% of the platelets analyzed)

The direct technique was employed according to the following procedure: platelets were incubated for 30 minutes at room temperature in the dark with the three anti-human Ig reagents; platelets from RBC group 0^+ healthy donors were analyzed as negative controls.[4]

Detection of serum anti-platelet antibodies was accomplished as follows: after fixation with paraformaldehyde, platelets from 0^+ donors were exposed to patient and control sera for 30 minutes at 37°C, washed twice and stained with the same three reagents.[5]

The three anti-human immunoglobulin reagents used in this study were: B-cell surface-immunoglobulin marker (Ortho Diagnostic Systems); total anti-human immunoglobulins/FITC (Ylem); rabbit anti-human K light chains/FITC and rabbit anti-human λ light chains/ RPE (Dakopatts).

Positive and negative controls

Serum from patients with transfused Cooley's disease, who were shown to carry platelet alloantibodies, and subjects with the proven presence of autoantibodies were utilized as positive controls; as negative controls we used serum from AB^+ healthy volunteers.

The platelet suspension, intended for both the direct and the indirect technique, was washed twice, resuspended in 0.5 mL of PBS and then analyzed with a FACScan flow cytometer (Becton Dickinson). Ten thousand cells were analyzed for each sample at a flow rate of approximately 200 particles per second. Data were registered as logarithmic fluorescence. The fluorescence intensity for the various anti-immunoglobulin reagents was determined for the platelet population by gating on light scattering parameters (forward scatter versus side scatter). Cells showing fluorescence intensities above the upper limit of the negative control distribution were considered positive. Patients with a percentage of platelet positivity higher than 15% for the anti-human monoclonal and polyclonal antibodies were considered to carry anti-platelet antibodies.[6]

Results

Table 1 summarizes the results obtained by using the indirect technique to measure anti-platelet antibodies in the serum. In brief, all the samples regarded as positive or negative controls were found to carry or not to carry, respectively, anti-platelet antibodies when examined by both flow cytometry and a standard light microscope. Five of 14 patients who showed clinical and laboratory features of chronic ITP were found to be strongly positive for anti-platelet antibodies, 3 of 14 were weakly positive, and the remaining ones were negative. The standard immunofluorescence method

Table 2. Surface anti-platelet antibody measurement by flow cytometry (direct technique).

	F C M		
subjects	+	±	–
0+ donors	0/36 (0%)	/	36/36 (100%)
ITP	5/14 (36%)	2/14 (14%)	7/14 (50%)
thrombocytop. states	8/22 (36%)	3/22 (14%)	11/22 (50%)

The results are expressed as number of positive cases and mean percentage of positivity (in parentheses).
Legend: FCM=flow cytometry; LM=light microscope; ITP=idiopathic thrombocytopenic purpura.
+ : strong positivity (> 20% of the platelets analyzed)
± : weak positivity (15-20% of the platelets analyzed)
– : negativity (< 15% of the platelets analyzed)

Table 3. Comparative evaluation of anti-platelet antibodies detected by flow cytometry using three different anti-human immunoglobulins.

	(indirect technique) type of anti-human immunoglobulins		
ITP (14 cases)	YLEM % (MFI)	ORTHO % (MFI)	DAKO % (MFI)
6 (–)	9 (123)	12 (130)	8 (121)
3 (±)	18 (209)	18 (225)	20 (210)
5 (+)	38 (262)	44 (279)	48 (266)

	(direct technique) type of anti-human immunoglobulins		
ITP (14 cases)	YLEM % (MFI)	ORTHO % (MFI)	DAKO % (MFI)
7 (–)	6 (85)	8 (104)	8 (65)
2 (±)	18 (207)	19 (218)	20 (214)
5 (+)	58 (232)	64 (261)	66 (266)

Data are expressed as mean percentage and mean of MFI (in parentheses).
Legend: ITP=idiopathic thrombocytopenic purpura; MFI=mean fluorescence intensity.
+ : strong positivity (> 20% of the platelets analyzed)
± : weak positivity (15-20% of the platelets analyzed)
– : negativity (< 15% of the platelets analyzed)

performed on a light microscope confirmed the flow cytometry findings in patients with chronic ITP, while some differences were observed in the other thrombocytopenic states, in which flow cytometry analysis detected a higher number of positive cases.

The data derived from determination of surface platelet antibodies is listed in Table 2. Briefly, in chronic ITP a marked positivity for anti-platelet antibodies was observed in 5 cases only, a weak positivity in 2 cases, and a complete negativity in the remaining patients, who showed mean fluorescence intensity identical to that of the negative controls. Flow cytometry analysis showed that thrombocytopenic patients affected by various hematological disorders were strongly positive in 8 cases, weakly positive in 3, and negative in the remaining 11 cases.

The number of positive cases was found to be similar for all three reagents used in this study, although the percentage of positive platelets was found to be higher for the k/λ monoclonal reagent (Table 3).

Discussion

Our data seem to support the sensitivity and reproducibility of the three techniques proposed herein, which were capable of identifying anti-platelet antibodies in all patients with transfused Cooley's disease who carried platelet alloantibodies, and in a significant number of patients with thrombocytopenic disorders including cITP.[7] Furthermore, all three reagents used in this study proved to be efficient tools for detecting anti-platelet antibodies both in serum (indirect method) or on the platelet surface (direct technique).

Our findings are in accordance with those derived from standard indirect immunofluorescence methods, which till now represented the most widely used techniques for detecting anti-platelet antibodies in thrombocytopenic states.

In conclusion, even though the monoclonal reagent (k/λ) proved to be the most reliable and sensitive under our conditions, due to the high quality of the fluorescence signal, nevertheless, all three methods presented here were capable of detecting anti-platelet antibodies in the patients studied.

Furthermore, a closer analysis of patients

showing dim fluorescence intensity revealed that flow cytometry displayed greater sensitivity than standard immunofluorescence, allowing us to speculate that the former could represent a useful means of detecting anti-platelet antibodies in various thrombocytopenic disorders.

However, based on currently available laboratory tests, decisive evidence for the presence of platelet auto-antibodies in thrombocytopenic patients is still not achievable since these procedures are not able to discriminate between platelet allo- and autoantibodies. Therefore the diagnostic power of these methods alone is not sufficient to explain the true autoimmune nature of these disorders.[8,9]

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