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Contribution of flow cytometry to the diagnosis and differential diagnosis of thrombocytopenia[1)]

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Abstract

Thrombocytopenia is commonly found in patients with apparent bleeding tendency, and in the laboratory evaluation of patients with other disorders or in clinically healthy individuals. Identification of the underlying cause is therefore crucial. There are several different laboratory tests available for this purpose. Flow cytometry employs very small sample volumes, which is of particular interest in patients with severe thrombocytopenia or in pediatric samples. Thus, the immunological determination of platelet numbers by flow cytometry is recommended as the reference method to achieve an exact quantification of very low platelet numbers, which could influence therapeutic decisions. In addition, flow cytometric analysis of immature platelets plays a role in the differentiation of increased platelet destruction and decreased platelet synthesis. As this parameter can be measured by current hematology analyzers, it will become more important in routine use. Another field for flow cytometry is the investigation of immunologically mediated thrombocytopenias. These can be caused by antibodies against platelet-specific glycoproteins such as autoimmune thrombocytopenia or by antibodies against heparin-platelet factor 4 complexes such as heparin-induced thrombocytopenia. Moreover, flow cytometric assays are used for the diagnosis of rare congenital thrombocytopenias, where they are either able to confirm a certain diagnosis, e.g., Bernard-Soulier syndrome, or at least establish a basis for further analyses. The contribution of flow cytometry to the investigation of lymphoma and leukemia, which regularly cause thrombocytopenia by bone marrow infiltration, is not discussed in this article.

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Introduction

Depending on the method used, platelet count in the peripheral blood of adults is approximately $150–450\times10^9$/L. As a rule no hemorrhagic bleeding exists with values $>100\times10^9$/L, even under hemostasiologic stress, and the routine clinical practice frequently speaks of thrombocytopenia only when values fall below this limit. When platelet values fall to $<30\times10^9$/L, the occurrence of spontaneous or serious cerebral and intestinal bleeding increasingly becomes a possibility [1].

A reduced platelet count can occur in a multitude of diseases which, in terms of pathogenesis, may be gathered into three main groups (see Table 1). 1) Decreased platelet production. Apart from the rare hereditary causes myelosuppressive drugs or displacement processes in marrow are the primary causes of decreased platelet production. 2) Increased platelet destruction and/or utilization. Here, the causes are immunologic mechanisms, such as auto-immune thrombocytopenia (ITP), which leads to a destruction of antibody-coated platelets in the spleen, as well as non-immunologic mechanisms like increased utilization, e.g., associated with disseminated intravascular coagulation (DIC). 3) Maldistribution. Normally 70%–80% of the blood platelets existing outside the bone marrow circulate in the peripheral blood, the remaining 20%–30% are stored in the spleen. This percentage can noticeably increase, e.g., in association with splenomegaly, and thereby lead to a lower platelet count. Based on these three main groups the causes behind a thrombopenia should be narrowed down further through targeted anamnesis (drugs, infections, etc.) and a thorough physical examination (splenomegaly, signs of bleeding etc.). Depending on the suspected cause this can be followed by specific further examinations (infection serology, bone marrow cytology, evidence of thrombocytic antibodies, etc.). In this connection, the domain of flow cytometry lies principally in the clarification of autoantibody induced immune thrombopenias, in the demarcation of decreased platelet production from increased platelet utilization through the identification of younger, immature blood platelets as well as in the identification of several rare hereditary platelet dysfunctions that accompany a thrombopenia. Before we discuss these areas in greater detail, we should note that prior to any further

Table 1 Differential diagnosis of thrombocytopenia (mod. after [2]).

1. Pseudothrombocytopenia (most often EDTA-induced)
2. Congenital thrombocytopenias
3. Acquired thrombocytopenias
3.1. Increased thrombocytolysis
• Immunologically communicated: ITP, neonatal alloimmuno thrombopenia, drug-induced ITP, post-transfusional, autoimmune diseases, lymphoproliferative disorders, HIV, HCV, Helicobacter pylori, HIT
• Non-immunologically communicated: stent, DIC, TTP/HUS, HELLP, eclampsia
3.2. Decreased neoformation
• Neoplasia: bone marrow infiltration, therapy associated (cytostatic agents)
• Virus infections: EBV, CMV, rubella, VZV, parvovirus B19
• Megaloblastic anemia
3.3. Distribution malfunction
• Hypersplenism

examinations – most of all with asymptomatic thrombocytopenic patients – an EDTA-induced pseudo-thrombopenia, which occurs with a frequency of approximately 1%, must be ruled out. This is an in vitro phenomenon in which the anticoagulant EDTA contained in the routine blood count tube causes agglutination of blood platelets, which then are no longer recognized by the automated blood count analyzer and therefore are identified as false-low. In a blood smear platelet aggregates usually appear as microcoagulation, less frequently as an accumulation to the leukocytes (''satellite phenomenon''). In order to obtain an exact platelet count and to allow a flow cytometric analysis, it would be advisable in such cases to use citrate tubes for blood collection or blood collection tubes specifically developed for this purpose (e.g., ThromboExact, Sarstedt).

An EDTA-induced pseudothrombopenia has no pathologic relevance, but should be known to both patient and physician, in order to avoid unnecessary diagnostics and possibly even treatments.

Immunologic determination of platelet numbers

Platelet numbers are determined in blood count analyzers mostly via the resistance measuring principle, which essentially classifies cells according to their size. In pathologic blood counts, which might contain microcytes, cell fragments or the like, this can mean that platelets can no longer be firmly demarcated, which can lead to an incorrect determination of platelet numbers. This is, of course, of particular relevance for patients with very low platelet numbers below 10,000 or 20,000/μL. The use of optical detection systems in modern hematology analyzers also could only improve but not satisfactorily solve the situation. The International Council for Standardization in Haematology (ICSH) therefore recommends as reference method the flow-cytometric determination of platelet counts following marking with platelet specific antibodies [3]. Here, the platelet count measured is placed in relation to the erythrocyte number measured in parallel. From this ratio and from the erythrocyte number determined at the blood count analyzer the absolute platelet number can then be calculated.

Reticulated platelets

When thrombopenia is diagnosed, the differentiation between abnormal production or increased consumption is of central importance. One possibility of obtaining information on the condition of the thrombopoiesis is to perform a bone marrow puncture with the necessary cytological and histological review. Far less invasive is the identification of young platelets in peripheral blood. These can be stained with high-affinity RNA colorants like thiazole orange and quantified by flow cytometry, whereby specificity can be heightened by means of fluorescence-marked platelet-specific antibodies [4, 5]. Although not applicable morphologically, these stained platelets are called reticulated platelets in analogy to reticulocytes, the immature precursors of erythrocytes. Meanwhile, some studies have shown that this parameter is useful in differentiating between consumptive and aplastic causes of thrombopenia [6, 7]. Even though the identification of reticulated platelets at the flow cytometer by means of fluorescence-marked antibodies must be considered the gold standard, this method is too costly to establish itself as a ''true'' routine parameter when clarifying lowered platelet counts. This gap could be closed by measuring this parameter at modern hematology analyzers. At present the devices manufactured by Sysmex offer the possibility of identifying the immature platelet percentage of the total number of platelets, expressed as immature platelet fraction (IPF). Measurement follows fluorescence staining in the reticulocyte duct. Forward-scattering light and fluorescence content are then analyzed in the flow cell. Various studies have shown that in patients with increased platelet consumption, e.g., within the context of autoimmune thrombopenia, IPF was increased or rather that an increase in IPF represents an early marker for a recovery of bone marrow function following chemotherapy or stem cell transplantation, so that, for example, control of the transfusion of platelet concentrates via this parameter would seem more conceivable as well [8–11]. Since other manufacturers of blood count analyzers are also working on automating the measurement of reticulated platelets and will introduce them in the foreseeable future (Siemens, personal notification), it can be assumed that – with routine availability – this parameter will find entry into basic diagnostics.

Auto-antibody induced thrombopenias

ITP is triggered by auto-antibodies directed against specific surface antigens of the platelets. The antibody-coated blood platelets are then increasingly destructed in the spleen and

thrombopenia ensues. Hence, it would stand to reason to draw on the identification of auto-antibodies, bonded to platelets or present and unattached in serum, for a diagnosis of ITP, although in practice this is clearly much more difficult. Older, but even today still used assays identify total immunoglobulin on platelets [platelet-associated IgG (PAIgG)]. However, besides auto-antibodies these tests also record IgG that is stored in the α-granules of blood platelets, bound to Fc-receptors and unspecifically immobilized on the platelet surface, which – depending on the test system used – leads to a sensitivity between 59% and 74% and a specificity between 19% and 74% [12]. The introduction of assays that no longer identify total, but only specific immunoglobulin directed against platelet glycoproteins resulted in a marked improvement of specificity. As early as the late 1980s monoclonal antibody-specific immobilization of platelet antigens (MAIPA) was developed as the new diagnostic test [13], which up to the present is considered the gold standard in platelet immunology [14]. Additionally, a series of flow cytometric assays was developed for the diagnosis of ITP and also to circumvent the limitations of MAIPA. The simplest method is the identification of a coating of the patient's platelets with IgG or IgM, which ultimately is comparable with the identification of PAIg in an immunofluorescence test. Theoretically, the specificity of antibodies can then be ascertained in a second step by using a glycoprotein-specific EIA [15]. For this platelet-bonded antibodies are acid-eluted and then put in the EIA. However, our practical experience with this test shows that positive samples as a rule exhibit an identical reaction pattern in the EIA and frequently react with several tested glycoproteins, so that the specificity of this method is at the very least questionable. Other flow cytometric assays attempt to furnish evidence of platelet-bound glycoprotein-specific antibodies in one step. In 1995, an assay was introduced that is based on the principle of the fluorescence-resonance-energy-transfer (FRET) [16]. Because it is not easily standardized this assay could not prevail in routine diagnostics [17]. Another assay was developed based on the flow cytometric identification of antibodies that react specifically with GPIIb/IIIa (CD41a) immobilized on microbeads [18]. Here, the platelet lysate was incubated with the coated microbeads and the antibody binding verified with a second fluorescein labeled polyclonal goat antihuman antibody. At the selected cut-off this study with 136 patients, of which 62 with ITP, resulted in a sensitivity of 86% at a specificity of 100%. A different approach was chosen with the simultaneous analysis of specific platelet antibodies (SASPA) assay [19]. Here, patient platelets are incubated with monoclonal mouse antibodies against various human platelet glycoproteins. The result is the formation of a termolecular complex consisting of glycoprotein, bound human antibody and bound mouse antibody. The platelets are then lysed and the termolecular complex is immobilized by binding it to beads coated with anti-mouse antibodies. Identification of bound auto-antibodies occurs via fluorescence-marked goat-anti-human antibodies. The assay was evaluated with patient serum as well as patient blood platelets that had shown a positive result in the PAIg-IFT. The resulting sensitivity and specificity were comparable to the MAIPA.

In spite of all the efforts to establish an easily performed and highly diagnostic assay for the identification of thrombocytic antibodies, the views on the valence of these tests in the diagnosis of ITP differ considerably, because of the poor standardization and the very heterogenic performance of the different test systems. While the guidelines of hematologic societies in the Anglo-American region do not recommend the identification of platelet antibodies [20], such identification is listed as one of the diagnostic procedures in primary diagnostics of thrombocytopenias in the guideline ''Thrombocytopenia'' of the Deutsche Gesellschaft für Hämatologie und Onkologie [1]. In summary, it can be said that while the identification of glycoprotein-specific auto-antibodies against platelets can corroborate a diagnosis of ITP due to the high specificity, an absent identification does not rule out an ITP due to the low sensitivity.

Heparin-induced thrombocytopenia

A further cause of an immunologically induced thrombocytopenia is heparin-induced thrombocytopenia (HIT). Because of the wide use of heparin and the serious and, at this time, life-threatening complications of HIT, ruling out this disease in patients in whom thrombopenia has been detected while receiving heparin therapy is of great importance. In the case of clinically suspected HIT, whose probability can be determined by means of the so-called ''4T-Score'' (Thrombocytopenia, Timing, Thrombosis and the absence of oTher explanations), antibodies against heparin-platelet factor-4 (PF4)-complexes as a rule are identified with immunological assays. Functional assays like the serotonin-release-assay or the heparin-induced platelet aggregation [21] are more specific, but also associated with higher technical and financial expenditure. Additionally, the last years saw the development of flow cytometric assays for HIT diagnostics. These detect an activation of blood platelets, for example, in that the serums of HIT patients lead to a release of procoagulatory platelet-microparticles (PMP) from normal donor platelets. Like anionic phospholipids these PMP can be identified flow cytometrically on the surface of activated platelets [22]. Recently, an interesting new method was publicized that combines the identification of heparin-PF4-antibodies with the functional identification of platelet activation [23]. Here, antibodies are identified with heparin-PF4-coated beads, platelet activation is determined by means of annexin-V-binding.

Thrombocytopathiae with thrombopenia

On the whole, hereditary platelet defects are rare. According to a current overview paper [24] a whole series of these diseases not only show a platelet malfunction but also a more or less pronounced thrombocytopenia. The underlying disorders of these diseases are very heterogenic, so that a broad

Table 2 Immunophenotype of selected hereditary thrombopenias (mod. after [24]).

Syndrome	Affected gene	Heredity	Phenotype	Immunophenotype
Bernard-Soulier syndrome (BSS)	GPIb-α, GpIb-β, GPIX	AR	Very large platelets, no aggregation can be triggered by ristocetin	Decreased expression of GPIb/IX/V on platelets
X-chromosomal inherited thrombocytopenia (XLT)	WAS	X	Small platelets, no immune defect	Identification of a decreased WASP-expression in PBMC
Wiskott-Aldrich syndrome (WAS)	WAS	X	Small platelets, immune deficiency, eczema, lymphoma	Identification of a decreased WASP-expression in PBMC
Congenital amegakaryocytic thrombocytopenia (CAMT)	c-MPL	AR	Congenital thrombopenia (normal size platelets), further cytopenias in the course	Identification of a decreased c-MPL-expression in platelets
Benign Mediteranean macrothrombopenia (MTCP) with coexpression of glycophorin A	GPIb-α, other?	AD	Enlarged platelets	Identification of a coexpression of glycophorin A on platelets

AR, autosomal-recessive; AD, autosomal-dominant; X, X-chromosomal; GP, glycoprotein; PBMC, peripheral blood mononuclear cells.

spectrum of different laboratory-diagnostic methods contributes to securing a diagnosis. In 2003, an algorithm for the identification of hereditary thrombopenias was introduced, which was then slightly modified in a follow-up study [25, 26]. Its focus is a thorough anamnesis – including family anamnesis – and the clinical examination of the patient, in order to recognize syndromic thrombocytopenias, e.g., associated with loss of hearing or skeletal deformities. Depending on the presumption diagnosis, these are then clarified further. Non-syndromic thrombopenias are classified according to the platelet size in a peripheral blood smear into microcytic, normocytic and macrocytic forms. If there are no further distinctive morphologic features, step-by-step diagnostics will then determine ristocetin-induced platelet aggregation, immunohistochemical staining of the H-chains of the non-muscle-myosin type IIA (NMMHC-IIA) in leukocytes as well as flow cytometric identification of the platelet glycoproteins GP Ib/IX/V. With this algorithm, it is possible to achieve at least a narrowing-down to a specific defect in many patients. Depending on the defect a diagnosis is subsequently secured through further specific examinations, such as SDS-PAGE, molecular genetic methods, electron microscopy or also flow cytometry. The diseases that can be diagnosed with this technology primarily include Bernard-Soulier syndrome (BSS), a rare, autosomal-recessive hereditary defect of the Willebrand receptor complex GPIb-IX-V [27], wherein various mutations in the GPIb-α, GPIb-β or GPIX genes can lead to a decreased expression of the receptor or to a dysfunctional receptor protein. The clinical picture is heterogenic, so that a slight tendency to bleed is just as possible as one that is life threatening. Heterozygote carriers can show a macrothrombopenia with no or usually mild symptoms. The flow cytometric diagnosis is established by identifying a decreased expression of CD42b (GPIb-α). Conformation-sensitive antibodies can even recognize qualitative defects. A significant problematic detail is that the absolute number of the GPIb-IX-V-complexes per cell on the enlarged platelets can be normal, while the receptor density is always decreased [25]. This can be detected by creating a ratio with another, properly expressed receptor like GPIIb or GPIIIa. Further flow cytometrically identifiable hereditary thrombocytopenias are the X-chromosomal hereditary thrombocytopenia [X-linked thrombocytopenia (XLT)] [28], the Wiskott-Aldrich syndrome (WAS) [29, 30], the congenital amegakaryocytic thrombocytopenia (CAMT) [31, 32], as well as the benign Mediterranean macrothrombopenia with thrombocytic coexpression of glycophorin [Mediterranean macrothrombocytopenia (MTCP)] [25]. An overview of the flow cytometric diagnostics of these diseases is summarized in Table 2.

Conclusions

During the last years a series of flow cytometric assays has been developed that can be used for the differential diagnostic clarification of a thrombopenia. Unquestionably, one of the advantages of this method is that as a rule only small sample volumes are required, which is of benefit mainly in the examination of pediatric specimens or in the diagnostic confirmation of patients with distinct thrombopenia. A further advantage surely is the possibility of simultaneously determining several parameters in one approach by using a combination of various fluorescence dyes or the use of beads. However, the latter is conditioned on a certain degree of complexity, which on the one hand places corresponding demands on both technical equipment and personnel of the performing laboratory, on the other hand at the very least complicates standardization and hence the comparability of the results of the laboratories involved. Standardization, however, is a prerequisite, if this method of analysis is to find wider distribution.

Additionally, it should be noted that for some of the diseases presented herein the value of flow cytometry is of minor importance or has no more than a complementary function. For example, although in cases of CAMT a diagnosis by means of flow cytometry is possible, a reliable diag-

nosis is ultimately provided by the molecular genetic identification of an MPL-mutation [33].

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