Flow Cytometry of Nonhematopoietic Neoplasms

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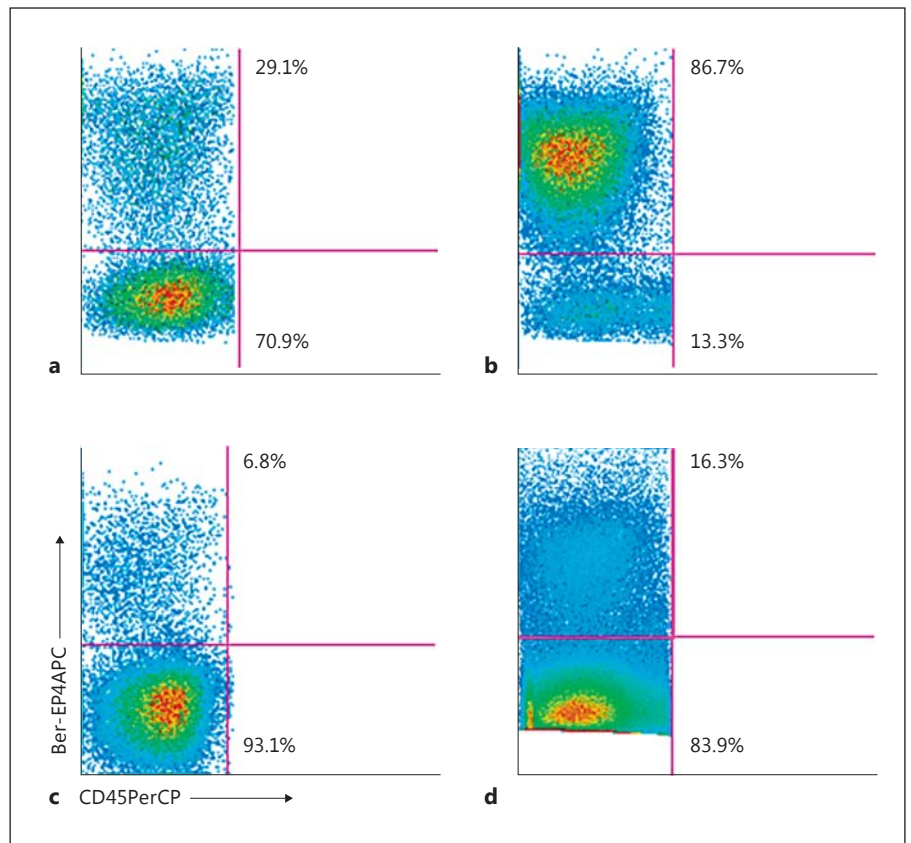
Abstract

Many epithelial neoplasms can be analyzed by flow cytometry (FC), particularly from serous cavity effusion samples, using EpCAM, a cell adhesion molecule expressed on most normal epithelial cells and expressed at a higher level in most epithelial neoplasms. A simple 3-color flow cytometric panel can provide a high sensitivity and specificity compared to cytomorphology. FC provides more rapid immunophenotyping than conventional immunohistochemical staining, can identify rare malignant cells that could be missed by a cytological exam alone, and can be utilized to evaluate limited samples such as cerebrospinal fluid or fine-needle aspiration samples. Flow cytometric analysis for epithelial antigens can be combined with DNA ploidy analysis or assessment of the nucleus-to-cytoplasm ratio. Panels of flow cytometric markers are useful for the assessment of pediatric nonhematopoietic neoplasms, including neuroblas-
tomas, primitive neuroectodermal tumors, Wilms' tumor, rhabdomyosarcomas, germ cell tumors, and hemangiopericytomas, as well as small-round-blue-cell tumors in adults, including small-cell carcinomas.

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Introduction

Flow cytometry (FC) is well established as a useful diagnostic modality for the assessment of hematopoietic neoplasms [1]. Increasingly it is also being used to detect low levels of disease in leukemias and lymphomas (minimal residual disease; MRD) [2]. FC is not used routinely in the diagnosis or follow-up of nonhematopoietic neoplasms. However, most nonhematopoietic neoplasms and tissues are amenable to flow cytometric analysis. FC can be especially helpful in serous cavity effusions and limited fine-needle aspirate (FNA) or cerebrospinal fluid (CSF) samples. Here we discuss the utility of FC in the diagnosis of epithelial neoplasms and small-round-blue-cell tumors in various samples. All research was conducted in compliance with institutional guidelines.

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Fig. 1. Ber-EP4 expression in various carcinomas present in malignant effusion samples assessed by flow cytometric analysis. **a** Breast ductal carcinoma. **b** Ovarian papillary serous adenocarcinoma. **c** Gastric adenocarcinoma. **d** Endometrial adenocarcinoma. Reprinted with permission from Pillai et al. [3].

Nonhematopoietic Neoplasms in Serous Effusions

Epithelial neoplasms are not commonly thought to be conducive to flow cytometric analysis, given their cohesiveness compared to hematopoietic neoplasms. However, we and others have shown that many common epithelial neoplasms can be easily analyzed by FC [3–6]. Serous cavity effusion samples, in particular, are very amenable to flow cytometric analysis since the cells are already dispersed in a cell suspension which includes single cells. While many antigens have been tested over the years, EpCAM (Ber-EP4, CD326, and MOC31) has emerged as the best marker to detect epithelial cells by FC [4]. EpCAM is a cell adhesion molecule [7] that is expressed on most normal epithelial cells in gastrointestinal, genitourinary, hepatobiliary, and breast tissue [8]. It is expressed at a higher level in most epithelial neoplasms [9]. EpCAM is not expressed on normal or neoplastic mesothelial or hematopoietic cells. Epithelial cells are not seen in normal serous cavity fluids, which typically contain mesothelial cells, monocytes/histiocytes, and lymphocytes. Hence, detection of epithelial cells is signifi-

cant in a patient with a history or suspicion of an epithelial neoplasm. Immunohistochemical staining for the presence of EpCAM in combination with cytological examination has a high sensitivity and specificity for the identification of malignant effusions [8]. Flow cytometric detection of EpCAM-positive epithelial cells shows a sensitivity and specificity comparable to those of immunohistochemical staining for the detection of malignant epithelial cells, and it can be used to detect a wide variety of epithelial neoplasms (fig. 1) [3–6]. In addition, FC is able to analyze a large number of cells compared to immunohistochemical staining, it is able to be performed rapidly, without tissue fixation and processing, and it can include a multiparametric analysis of multiple antigens simultaneously.

The gold standard for the diagnosis of malignant effusions is a cytological examination. However, it is known that cytology has a high specificity but a lower sensitivity [10]. Frequently, multiple samples are needed for a definitive diagnosis. We and others have shown that EpCAM-based FC has a greater sensitivity than cytology [3, 11–13]. In addition, FC can provide rapid confirma-
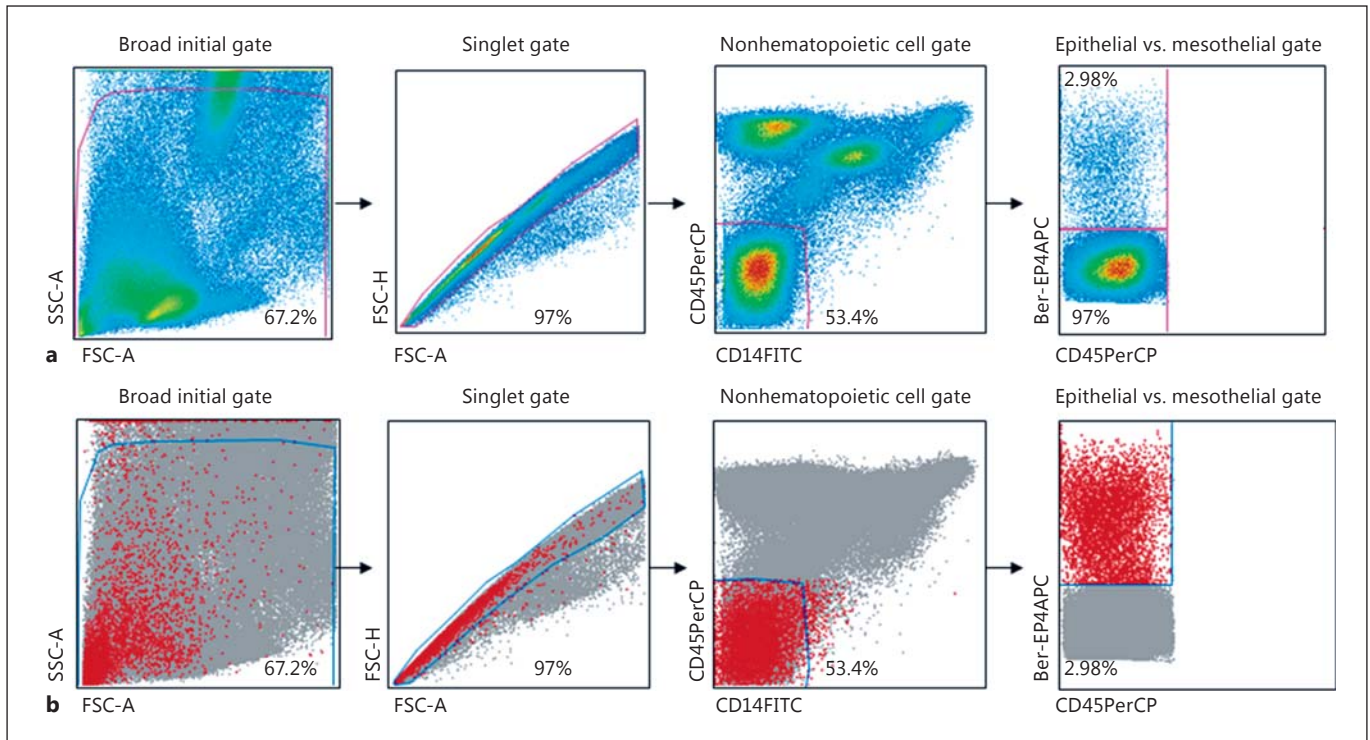
Fig. 2. Flow cytometric gating strategy for epithelial neoplasms. The majority of cells in the forward vs. side scatter gate should be included in the analysis for maximum sensitivity. Use of the singlet gate eliminates clusters of cells that may complicate the interpretation of subsequent gates. CD45 and CD14 are used to exclude hematopoietic cells and monocytes. Ber-EP4 (EpCAM)+ cells are epithelial cells. The dot plots in **b** are the same as those in **a**, but they are back-gated to show the locations of Ber-EP4-positive cells in all of the plots. Reprinted with permission from Pillai et al. [3].

tory immunophenotyping even in cases that are positive by morphology, obviating the need for immunohistochemical staining. Also, cytological examinations are sometimes reported as suspicious or atypical based on the presence of rare cells that are not sufficient for a positive diagnosis. For clinical decision making, these findings are considered negative. FC can rapidly analyze a larger volume of fluid in such cases, and it is even possible to analyze the entire volume of fluid. Hence, rare malignant cells can be detected using this method and these cases can now be called positive with a greater degree of confidence. In addition to improving the turnaround time for a positive result, FC can obviate the need for multiple paracentesis procedures.

The flow cytometric gating strategy to analyze epithelial cells in effusion specimens is relatively straightforward (fig. 2). First, CD45-positive events are excluded from the analysis to eliminate hematopoietic elements such as lymphocytes and monocytes that are frequent components of effusions [3]. That leaves mesothelial cells and any epithelial cells in the analysis, which are then distinguished from each other by an epithelial marker. While most studies use purely quantitative criteria, we believe that it is important to use a combination of quantitative and qualitative criteria to make a diagnosis of malignant effusion [3]. Additional epithelial or mesothelial markers can be used to increase the specificity but they are not essential. We have shown that a simple 3-color FC panel can provide a high sensitivity and specificity compared to cytology [3]. However, it is important to note that, while hematopoietic elements occupy well-established regions in the forward versus side scatter plot, epithelial elements are variable in size and cytoplasmic complexity and may be present in any region of the forward versus side scatter plot. Hence, all flow cytometric events should be included in the initial analysis, including the debris region (fig. 2). We have found that many of the EpCAM-positive events are in the debris region, but they are not necrotic or dead cell debris since they do not bind other antibodies nonspecifically [3]. We hypothesized that these events repre-

sent fragmented tumor cells. However, it was recently determined that these are most likely EpCAM-positive microparticles, which are extracellular vesicles containing antigen from the parent cell [14]. They have been found only in malignant effusions and their detection can improve the sensitivity and specificity of the cytological examination. We have also seen some tumor cells in the CD45-positive fraction in cases with large numbers of tumor cells. We hypothesized that these are tumor fragments that have adhered to or been endocytosed by hematopoietic elements. Again, the presence of EpCAM-positive micoparticles can account for these events as well.

One pitfall of using EpCAM alone for flow cytometric analysis of epithelial neoplasms is that a small proportion of epithelial neoplasms do not express EpCAM [8]. However, as is well known with immunohistochemistry, multiple antibodies are needed for improved sensitivity and specificity. Given the increasing prevalence of multiparameter FC, it is relatively straightforward to add more antibodies to the panel. Other antigens that have been tested for the detection of epithelial cells by FC include claudin 4, B-72.3, EMA, CEA, and CD15 [4, 15]. Another pitfall of EpCAM-based FC is that EpCAM-positive events can rarely be found in negative serous effusions and lymph nodes in very low numbers, and it is not clear at present whether these represent nonspecific background staining or true epithelial cells. Nonspecific staining can be identified by its pattern of flow cytometric staining (dim, scattered rather than clustered, diagonal 'rocket' population), but it is sometimes difficult to differentiate it from true low-level staining. Benign epithelial cells can sometimes be present in lymph nodes and effusions [16]. Mesothelial cell inclusions have been noted in lymph nodes, but mesothelial cells are negative for EpCAM and positive for other keratins [17]. In contrast, specimens such as bronchoalveolar lavage and peritoneal washes always contain normal EpCAM-positive epithelial cells that are difficult to distinguish from malignant cells by FC. Most studies exclude these samples from their analysis for that reason.

One approach to distinguishing benign from malignant cells by FC is through DNA ploidy analysis since tumor cells frequently show a polyploid or aneuploid tumor content compared to benign cells that have a normal, diploid DNA content [18]. Numerous studies have shown that DNA ploidy has a predictive and prognostic value in epithelial tumors. Image cytometry is suggested as a better way to measure DNA ploidy because it can combine morphological analysis with ploidy [19]. However, FC

has the advantage of analyzing surface and cytoplasmic markers concurrently, which can help to identify the malignant cell population accurately. Measurement of the DNA content in isolation without any epithelial markers has not been found to increase diagnostic specificity [11–13]. Another promising approach to distinguishing benign from malignant cells is the use of flow cytometric measurement of the nucleus-to-cytoplasm (N:C) ratio. Automated FC based on the DNA content and the N:C ratio shows a high specificity (and similar sensitivity) compared to the HPV Hybrid Capture 2 system in the analysis of cervical cytology samples [20]. Another approach for the identification of malignant cells is the use of antibody-coated magnetic beads. The commercially available circulating tumor cell assay system CellSearch® from Veridex essentially combines EpCAM magnetic bead sorting with flow cytometric detection of cytokeratin-positive events and visual confirmation of positive events. Newer circulating tumor cell assays have tried to develop cell size- and cell property-based methods that do not utilize EpCAM given that some epithelial neoplasms may be EpCAM negative.

The expression level of EpCAM has a prognostic significance in breast and ovarian cancers [8]. The EpCAM- and CD3-targeting biantibody catumaxomab is effective for the treatment of malignant ascites due to ovarian cancer [21]. The anti-EpCAM bispecific monoclonal antibody solitumomab is in clinical trials for solid tumors [22]. Flow cytometric assessment of EpCAM expression could be useful in determining the level of EpCAM expression and its correlation with treatment response when these therapeutic antibodies are used.

Nonhematopoietic Neoplasms in Lymph Nodes

Lymph nodes are similar to effusions in that they normally lack epithelial cells. Hence, the flow cytometric approach detailed above can also be used to detect malignant epithelial cells in lymph node biopsies or FNA specimens (useful antigens are summarized in table 1). In many cases, FC is routinely performed on lymph nodes to rule out lymphoma and an additional analysis for EpCAM-positive cells can be easily added to the analysis if an epithelial malignancy is in the differential diagnosis [23]. The flow cytometric panel can be further extended to identify other solid tumors in tissues as well. Finely mincing tissue will yield sufficient cells for immunophenotypic analysis in many cases. Enzymatic digestion has been suggested to improve the yield, but it can also cleave

Table 1. Typical flow cytometric findings in specific nonhematopoietic neoplasms

Neoplasm	Flow cytometric findings	Ref.
Most carcinomas	EpCAM+, CD45–, CD14–	3, 23
Small-cell carcinoma	EpCAM+, CD56+, CD71+, CD45–	23
Merkel cell carcinoma	CD56+, CD71+, CD45–	23
Neuroblastoma	CD56^{hi}, GD2+, CD81+, CD9+, CD45–	24, 35
Rhabdomyosarcoma	MYOD1+, myogenin+	24
Germ cell tumors	CD56+, NG2+, CD10+, CD45–	24
Hemangiopericytoma	CD34+, CD45–	24
Primitive neuroectodermal tumor/Ewing sarcoma	CD56+, CD271+, CD99+	24
Wilms' tumor	CD56+, multiple populations	24

surface antigens of interest and is not recommended. Tissue homogenizers that are used in routine FC may fragment the tumor cells and need further investigation. A pediatric cancer FC panel that was used on a wide variety of tissue samples revealed distinct immunophenotypes for neuroblastoma (CD56hi/GD2+/CD81hi), primitive neuroectodermal tumors (CD271hi/CD99+), Wilms' tumor (CD56+, multiple populations), rhabdomyosarcoma (MYOD1+/myogenin+), germ cell tumors (CD56+/CD45–/NG2+/CD10+), and hemangiopericytomas (CD45–/CD34+) [24]. A high concordance was demonstrated between morphological and immunohistochemical findings and flow cytometric characterization. Although subclassification and a definitive diagnosis require morphological assessment, the advantage of flow cytometric analysis in this situation is that it provides a rapid, same-day immunophenotypic characterization. As is the case in the characterization of hematopoietic neoplasms, FC adds to the morphological diagnosis and is not meant to be a stand-alone technique since there will be neoplasms that cannot be detected or fully characterized by flow cytometric analysis alone. Another caveat is that rare malignant cells in lymph nodes may be lost during processing or may not be at a level higher than the background. Hence, FC should be used only for characterization of lymph nodes that are clearly involved by tumor and not for sentinel node assessment, for example.

Flow Cytometric Evaluation of CSF Samples

Leptomeninges are a frequent site of metastasis, and CSF examination is routine in hematological malignancies. CSF samples are unique given the limited amount of fluid available to make a definitive diagnosis. Lymphocytes and monocytes are the predominant cells found in CSF, and any aberrant cells present can be identified in cytological preparations. Cytological examination is the gold standard for diagnosis, but a proportion of patients can have false-negative results. Similar to effusions, multiple specimens are needed for a higher sensitivity [25]. As is the case with serous effusions, frequently there are too few cells that can be definitively identified by morphological or immunohistochemical assessment. Furthermore, an early diagnosis is needed in order to initiate definitive treatment and limit neurological morbidity. FC is a sensitive technique for the detection of abnormal lymphoid populations in CSF [26, 27]. Flow cytometric analysis shows a high sensitivity and specificity for the diagnosis of carcinomatous meningitis [28, 29]. The utility of FC lies in the ability to immunophenotype rare cells. Rapid degeneration of CSF specimens, a low volume and cellularity, blood contamination, and nonspecific background fluorescence are technical issues that need to be resolved [26]. It is suggested that at least 5 ml of fluid and 100 cells need to be analyzed, with a cluster of at least 10 events in order to consider a result positive. RPMI-based serum solutions of Earle's balanced salt solution with albumin can be used to prevent cell degeneration in CSF specimens. Minimization of cell washing steps and gentle centrifugation are helpful as well. Performance of all of the steps in microcentrifuge tubes can also minimize cell loss. Nonspecific fluorescence can limit the sensitivity of the assay and can be minimized by appropriate color combinations and titration. A similar approach can also be used in limited FNA samples.

Flow Cytometric Evaluation of Small-Round-Blue-Cell Tumors

FC is useful in the differential diagnosis of small-round-blue-cell tumors. CD56, CD99, and myogenin can be used to subtype these tumors [9, 23, 24]. CD56 is a neural cell adhesion molecule that is expressed on NK cells and neurons. CD56 is expressed by tumors of neural origin, including neuroblastoma and small-cell lung cancer (fig. 3, 4). CD56 is widely used in FC labs for immunophenotyping of large granular lymphocytes and NK cells, and hence it can be easily adapted to detect nonhematopoietic tumors as well. Neuroblastoma is also positive for CD81, CD9, and GD2 [9]. GD2 is a disialoganglioside that is normally expressed in the brain and overexpressed on tumors of neuroectodermal origin.
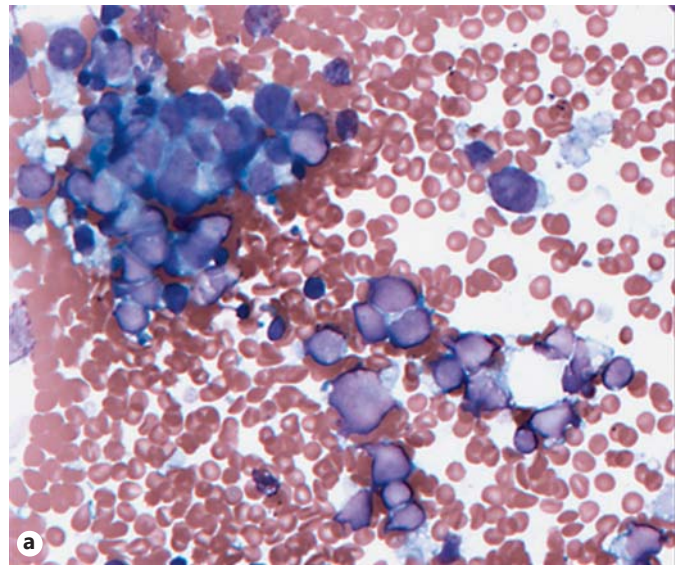
Fig. 3. Representative flow cytometric analysis of small-cell carcinoma. A 45-year-old man presented with shortness of breath, facial swelling, weight loss, fever, and a right hilar mass involving the superior vena cava, and he was suspected to have an aggressive lymphoma. **a** FNA revealed the presence of cells with a high N:C ratio, present individually and in clusters. **b** By flow cytometric analysis, the neoplastic cells were found to be negative for hematopoietic cell markers, including CD45, B-cell, and T-cell markers (data not shown), but they were positive for CD56, but not CD16, consistent with small-cell carcinoma.
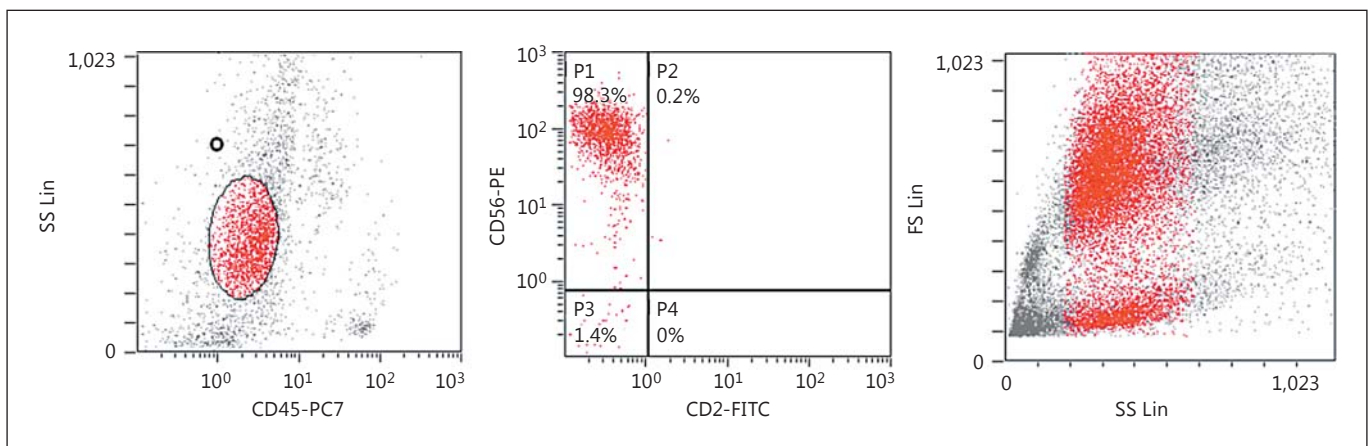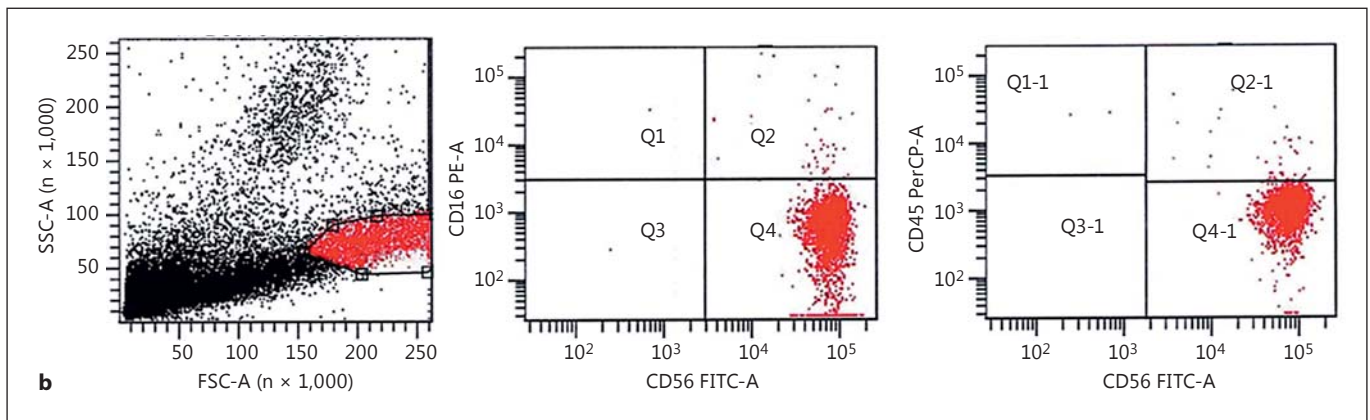
Fig. 4. Representative flow cytometric analysis of a neuroblastoma. Flow cytometric analysis was performed on a bone marrow aspirate from a 6-year-old boy with an adrenal mass. A large population of CD45-dim-to-negative, CD56-positive cells is present, which is negative for CD2 and other lymphoid and myeloid markers (data not shown), consistent with involvement by a nonhematopoietic tumor. The image is courtesy of Dr. Michele Paessler.

Myogenin and MYOD1 are muscle-specific transcription factors that are expressed by a majority of rhabdomyosarcomas [30]. CD99 is a cell surface glycoprotein that is normally expressed by cortical thymocytes and some epithelial cells and it is frequently used to identify lymphoblastic lymphoma/leukemia and Ewing sarcoma/primitive neuroectodermal tumors [9].

FC has been utilized to detect neuroblastoma in bone marrow specimens [31]. Approximately 50% of patients with neuroblastoma have bone marrow metastases at diagnosis, which is associated with a poor overall survival [32]. CD56 and GD2 are useful markers for flow cytometric detection of neuroblastoma. Although neuroblastoma occurs as clusters in bone marrow aspirate preparations, frequently there are admixed single cells that can be detected by routine FC. Not infrequently, lymphoblastic leukemia is in the differential diagnosis, and neuroblastoma is identified by the presence of a CD45-negative, CD56-positive aberrant population (fig. 4). FC has also been used to detect MRD after treatment in neuroblastoma patients [33]. The sensitivity depends partly on the total numbers of events collected. For example, routine FC, in which up to 100,000 events are analyzed, can detect 1 in 10,000 cells or 10–20 positive events. MRD flow cytometric analysis for the assessment of acute leukemia MRD routinely analyzes 1–5 million events and has a higher sensitivity compared to routine FC [2]. GD2 immunocytology and tyrosine hydroxylase RT-PCR have also been used to detect MRD in neuroblastoma, and they are thought to have a higher level of sensitivity (i.e. 1 in 1,000,000 cells) [34, 35]. However, immunocytochemistry and RT-PCR are time-consuming and labor-intensive techniques. Multiparametric MRD flow cytometric analysis has not been tested rigorously in the assessment of bone marrow samples involved by neuroblastoma, and it may be able to detect low-level disease similarly to immunocytochemistry and RT-PCR.

Future Directions

FC technology is rapidly evolving. Mass spectrometry-based flow cytometers that use heavy metal ions rather than fluorochromes are being used in research applications. This has enabled simultaneous measurement of as many as 40 immunophenotypic parameters [36, 37]. Preliminary results are promising for the characterization of clinical samples involved by hematopoietic neoplasms. Manual gating is difficult with such technologies and automated gating strategies are proposed to analyze complex multiparametric data [38]. Molecular techniques are increasingly being used for clinical diagnosis, but they are sometimes limited by low levels of disease involvement. The combination of flow cytometric cell sorting and a subsequent molecular analysis will be helpful in obtaining additional diagnostic information from clinical samples with low-level disease or limited FNA or body fluid samples. Information about clonal evolution and medically relevant subpopulations can also be obtained through flow cytometric cell sorting.

Disclosure Statement

The authors have no conflicts of interest to report.

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