Review paper

Flow cytometry as a reliable tool in diagnostics – review of basic principles, standard procedures and tests in diagnostics of primary immunodeficiencies

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Abstract

The paper presents basic principles in application of flow cytometry in diagnostics of primary immune deficiencies.

Key words: *primary immune deficiencies, flow cytometry, application guidelines.*

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Introduction

Flow cytometry is nowadays a very popular diagnostic method used in various fields of medicine. The technology allows detection of any molecule (antigen) on the cell surface or inside it, if an antibody against this molecule is available. These molecules are actually functional proteins involved in cell communication, adhesion or metabolism. Changes in cell metabolism and cell cycle may be also detected due to changes in light emission of stains caused by changes of conditions within the cell.

It shall be remembered that despite common technology there are different approaches to its use depending on the field of application. To make the analysis reliable for diagnostic purposes several guidelines have been developed in the past. In most cases they relate to application of flow cytometry to hematooncology [1-3], with relatively few referring to immune deficiencies, among them mostly to secondary immunodeficiencies due to the growing population of HIV infected patients [4-6]. There are few papers addressing systematically the philosophy and methodology of flow cytometry applied to diagnostics of primary immune deficiencies. This review, based on several guidelines [6-8], as well as author's own experience is aimed at presentation of systematic approach to flow cytometry as a safe and reliable tool, reminding basic safety principles, and presentation of tips specific to diagnostics of primary immunodeficiencies.

Purpose of immunophenotyping

Normal lymphoid system develops with time, with various molecules appearing on and disappearing from the cell surface or the cell's interior. Changes in relative and absolute values of individual cell populations specific for particular age ranges allow definition of absent or abnormally numerous cell populations. Depending on other features such picture might be specific for immunodeficiency or malignancy. In immunophenotyping of leukemia or lymphoma basic objectives include distinguishing neoplastic cells from normal elements and description of antigenic profile of abnormal cells [9]. In the diagnostics of primary immune deficiencies the basic goal is to enumerate particular cell types, to evaluate their function or to detect a particular gene product. Information gathered using flow cytometry together with other clinical and laboratory data help in making decision on further testing, especially genetic testing, required to establish definite diagnosis [8].

Primary immune deficiencies affect mostly children. Having in mind that the immune system develops with

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subject's age, with dynamic changes in lymphocyte subpopulations, it is very important to refer to age-related normal values for relative, as well absolute cell counts. Such normal age-related values for basic lymphocyte subpopulations are available in the literature [10-14] and for wider scope of cell populations are under development as part of research carried in the Children's Memorial Health Institute, Warsaw, Poland.

Laboratory safety

As biologic specimens can harbor unknown pathogens that may be infectious to laboratory personnel it is important to consider all samples as hazardous to health. Although common bloodborne infectious agents as Human Immunodeficiency Viruses 1 and 2 and hepatitis viruses are primarily transmitted through inoculation, they may also be transmitted through mucous membrane exposure. Detailed discussion on laboratory procedures regarding sample and equipment handling is beyond the scope of this paper, but universal biosafety rules shall be adopted in the laboratory and all manipulations shall be done by staff protected from exposure to accidental splashes or inoculation [15]. Flow cytometer manufacturer's instructions shall be followed in disinfection of the machine's fluidics. It shall be assured that all samples are properly fixed after staining, but before analysis. Some commercial lysing/fixing reagents reduce infectious activity by several logs, but in most cases they are not evaluated for their effectiveness in this regard [16], therefore despite fixation the samples shall still be considered as infectious.

The sample

Appropriate type of sample shall be selected for analysis. Selection of anticoagulant for flow cytometric immunophenotyping will depend mainly on anticipated delay before sample processing and type of tests carried during phenotyping. Standard surface staining is usually carried on samples anticoagulated with K_3EDTA (1.5±0.15 mg/ml blood), acid citrate dextrose (ACD) or heparin, if specimens are processed within 30 hours after collection [16]. If the specimens will be processed within 48 hours after specimen collection, ACD or heparin should be used. Specimens that were not processed within 48 hours after collection shall be rejected [17]. Type of anticoagulant may change when the analysis requires stimulation. In such cases usually heparin is used. One shall remember that stimulation effect may be lower when lithium heparin instead of sodium or preservative – free heparin is used (unpublished observation). In any case the sample shall be mixed well after drawing to prevent clotting.

Samples shall be properly labeled with unique patient's first and last name and time of blood drawing, to avoid inappropriate ascribing of results. They shall be accompanied by clearly completed written request for testing and maintained and transported before processing in room temperature (18-22°C). Extreme temperatures shall be avoided: temperatures above 37°C may cause cellular destruction and affect measurements [18]. The effect of low temperature (around 4°C) is unclear, but frequently loss of cells is observed. Therefore the optimal storage and transport conditions must remain between 10°C and 30°C and the samples should not be subjected to temperatures below 10°C [19, 20]. Sample integrity shall be analyzed immediately after delivery – specimens hemolyzed or frozen, clotted or older than 48 hours shall be rejected, just as samples that do not meet other requirements for reliable analysis.

In early 1980-ties determination of lymphocyte subsets was usually undertaken using lymphocytes isolated on density gradient, despite the fact that as early as in 1980 it was found that simple and rapid measurement of lymphocyte populations from very small samples of peripheral blood by immunofluorescence is feasible [21]. By early 1990-ties it was demonstrated that separated mononuclear cell preparations exhibit selective loss of specific cell populations. Such differences were noted in samples from normal individuals, as well as in specimens from human immunodeficiency virus infected individuals or with malignancies [22, 23], making this type of procedure unacceptable for samples in which exact relative and absolute counts are of utmost importance as it is the case of immunodeficient patients. At present there is no rationale for using either peripheral blood or bone marrow samples separated by gradient centrifugation. Considering numerous reasons for avoiding arbitrary loss of specific cell populations and great reduction of work time and workload whole blood or whole bone marrow phenotyping techniques shall prevail [24]. It shall be remembered that light scatter characteristics depends on the type of red cell lysis solution and type of immunofluorescent staining [25] and any interpretation shall refer to normal values established using equivalent antibodies, lysing solution, and equipment. If however, for whatever reason, cells need to be isolated or a lyse-wash technique must be used some source of protein, e.g. bovine serum albumin or fetal bovine serum, shall be used in the wash buffer to reduce cell clumping and non-specific fluorescence [16].

Single versus double platform strategy

Enumeration of absolute cell numbers and their subsets in clinical samples is of primary importance in HIV-infected patients, candidates for autotransplantation, or in evaluation of leukoreduced products [26], but it is equally important in the diagnostics of immune deficiencies. There are two strategies that help addressing this issue: double – or single platform strategy. Double platform strategy means that cell count is determined on hematological analyzers in separate samples, which shall be in such case drawn simultaneously with the sample used for immunophenotyping. This approach requires collection of two samples, which is sometimes very

difficult in pediatric patients most frequently diagnosed for immunodeficiency and, from obvious reasons, it is less accurate.

The so-called single platform strategy allows determination of cell counts in the same specimen that is submitted to immunophenotyping. This is done by adding standardized bead suspension to the immunophenotyped sample. Major suppliers of flow cytometers and monoclonal antibodies used for flow cytometry provide the users with such beads, usually free of charge when purchased together with monoclonal antibodies. Whatever type of beads is used they shall be mixed well to achieve final concentration of approximate 1000 beads per microliter of original sample, the sample cell concentration shall be not higher than 30.000 cells per microliter, and the total cell/bead event ratio shall not be greater than 10 [26]. This approach requires application of the gating strategy based on discrimination of cell populations by side scatter properties and expression of CD45 – this issue will be addressed later in this paper. If the user is not equipped with special software that allows direct calculation of absolute cell counts, a formula presented below shall be used [7]:

$$\frac{\text{number of events in CD45 bright region}}{\text{number of events in microfluorosphere region}} \times \frac{\text{total number of microfluorospheres added}}{\text{volume of blood added}}$$

Whenever single platform strategy is used reverse pipetting technique with precisely calibrated pipettes shall be applied to improve precision and accuracy of analysis. The beads are usually optimized to work in pre-defined conditions, therefore manufacture's instructions shall be strictly followed.

Staining conditions

Cells may be stained directly with a monoclonal antibody labeled with a fluorochrome or indirectly, with a primary antibody followed by a secondary antibody labeled with a fluorochrome. Antibodies interact with cells by binding specifically to the respective epitope, to Fc receptors, and by non-specific binding, so the selection of the antibody shall be carefully considered. With indirect labeling any secondary antibodies bound to fluorescent dyes shall be F(ab')2 fragments to eliminate Fc binding. Every antibody shall be titered prior to use so that correct amount is used to achieve optimal staining, despite the fact that commercially available antibodies are usually titered by the supplier for typical applications.

Cells stained for expression of surface or intracytoplasmic immunoglobulin light or heavy chains must be extensively washed of serum prior to adding antibodies. Staining shall be performed in plain phosphate buffered saline whether a blocking agent is applied or not, otherwise false negative results are probable [27].

Control of autofluorescence shall also be analyzed, using a sample without an added antibody and processed in the same way as the remaining samples. Ideally autofluorescence control shall give the same result as the isotype control used to evaluate the extent of non-specific binding.

As it was mentioned previously there is not need to isolate lymphocytes from whole blood or bone marrow specimens – for analysis of this type of samples whole blood or bone marrow lysing method shall be applied to remove erythrocytes. Specimens obtained from lymph nodes or plural fluid do not need to be lysed. Several commercially available lysing reagents usually contain also fixative in adequate concentration. It is also possible to use home-made lysing agent and fixative, usually a buffered ammonium chloride solution and a paraformaldehyde solution. It shall be remembered that these solutions have to be prepared fresh daily, as dissolved CO_2 in water combines with NH_4Cl forming carbonate $(NH_4)_2CO_3$ ineffective in process of lysing erythrocytes. Paraformaldehyde dissolved in aqueous solution becomes equivalent to ultrapure formaldehyde and is available from several companies labeled as useful for flow cytometry. It is not recommended to use for fixing cells paraformaldehyde that was not prepared specially for flow cytometry, because autofluorescence of cells can markedly increase. For some purposes fixation with cold pure acetone or methanol may be recommended [28].

Optimal results of cell surface staining are achieved when the procedure is performed within 30 hours from collection, but not later than 48 hours after drawing the blood specimen [29, 30]. Any centrifugation steps shall apply centrifugation forces not greater than 400 g for 3-5 minutes for wash steps.

Sample tubes shall be vortexed to mix blood with reagents and to break cell aggregates, as well as immediately before analysis to achieve best dispersion of cells. All incubations shall be carried in the dark, for period determined by manufacturer of the monoclonal antibodies or as defined by the user. Usually 15 minutes incubation time in room temperature or 30 minutes in 4°C is sufficient to achieve good staining results. Acquisition to flow cytometer in most cases can be done within 24 hours from staining, with the samples stored in refrigerator (4-10°C) until analysis.

How many colors

To accomplish the objective of phenotyping irrespective from its purpose it is essential to employ multiparameter analysis, as only through combination of parameters normal and abnormal cell populations may be reliably described. This shall be at least four parameter analysis, i.e. defining cells in terms of size (forward scatter) and granularity (side scatter), with at least dual color fluorescence [9]. Nowadays analysis of least 3-4 color fluorescence is preferable and for many applications high-level multicolor flow cytometry may offer the possibility of an increased accuracy [31].

Isotype controls shall be used when monoclonal antibodies label populations that do not have clear negative

results. Cursors on isotype control shall be set to include less than 2% of positively stained cells. Whenever clear labeling, as in case of staining for CD3, CD4 and CD8 at the same time or whenever multicolor analysis is applied including analysis of populations by definition negative in specific staining, isotype control is not necessary. In such case labeling with these antibodies result in fluorescence patterns in which unlabeled cells are clearly separated from the labeled ones, negative cells on the histogram offer an appropriate isotype control, and cursors set on these stainings may be used for the remaining tubes. Some adjustment is possible for more accurate separation of populations, unless the fluorescence distribution pattern is continuous, with no clear demarcation between positive and negative labeling [7]. Probability of proper identification of abnormal population in a heterogenous sample is greater when more complex analytical process is applied.

Gating strategy

Selection of an appropriate cell population for analysis is a fundamental analytical strategy called gating. Determination of gated population depends on the purpose of immunophenotyping and it will be different for various diseases. It is well recognized, that relying solely upon light scatter characteristics may lead to problems with contamination, therefore universal gating strategy for all samples, employing forward vs. side light scatter will not be appropriate, despite its primary use for definition of lymphocytes. Technological advances including developments in software and availability of monoclonal antibodies labeled with various fluorochromes facilitated identification of lymphocytes first using light scatter characteristics and since middle 1990-ties by defining lymphocytes on the basis of specific CD45 expression pattern and light scatter characteristics [5]. Today it is a common and recommended practice to employ CD45 as a third color for identification of lymphocytes being cells with low scatter and bright CD45 expression [5, 32-34], although some authors still recommend forward and side scatter gating for primary gate and use of CD45 in second line of analysis [35]. It may be used for both pediatric and adult specimens, although some more technical expertise is required for machine set-up and data analysis [5]. In any case it is important that initial evaluation shall include investigation of viable cells.

Acquisition

The subject of proper instrument set-up is far beyond the scope of this paper and has been addressed by manuals of instrument manufacturers and many papers [7, 36]. The choice of gating strategy will influence the method of analysis. In routine lymphocyte immunophenotyping it is accepted to evaluate at least 5000-10000 cells [37]. It is preferable that the data are acquired not gated and a minimum of 2500 lymphocytes are collected. This condition is especially important in case of potentially immunodeficient patients, who may have very low lymphocyte counts. For whatever reason data is acquired gated the gate setting shall be checked for each tube in order to adjust for tube-to-tube variation [5]. The gated cells can be subsequently used for various fluorescence parameters.

Definition of cell populations

Recognition of abnormal cells in tissues or body fluids is based on the knowledge of light scatter and immunophenotypic characteristics of normal cells present in those sites. It is not appropriate to use a nonspecific isotype control to set a threshold and then calculate percent positives by applying this threshold to histogram of stained population. This approach may be appropriate when the gated population is composed only of cells of interests and if the shape of fluorescence distribution clearly discriminates between positive and negative populations. In general, for hematooncologic purposes qualitative discriptors such as positive or negative are more useful and informative. In case of immunodeficiencies precisely calculated absolute counts of individual cell populations may decide on the diagnosis.

Before any further analysis is done on a whole blood sample, irrespective of the purpose of phenotyping, it is wise to verify distribution of main leukocyte populations. This additional step may bring important information, such as low or high leukocyte counts or presence of populations with abnormal scatter characteristics, and help in avoiding unnecessary staining in case of absent populations (own observation). Verification of CD45 fluorescence distribution on lymphocytes may also help to determine the presence of abnormal cell populations (malignant cells, transfusion – derived cells etc.).

For whatever reason phenotyping is carried monoclonal antibody panels must contain appropriate antibody combinations to enumerate cells. T cells shall be defined as CD3+, NK cells as CD3 negative, CD16 and/or CD56positive, and B cells as CD19+ or CD20+. T cell helper subpopulations shall be defined as CD3+and CD4+, T suppressor/cytotoxic cells shall be defined as CD3+ and CD8+ and they shall be reported as percentage of total lymphocytes [5]. Optimally the sum of percentage of T, B and NK cells in the lymphocyte gate (the so-called lymphosum) shall be between 95-105%, (or at a minimum 90-110%) [16, 38]. Optimally the sum of the percentage of CD3+CD4+ and CD3+CD8+ cell should be equal to the total percentage of CD3+ cells plus or minus 5%, with maximum reliability equal or less to 10%. In specimens containing considerable amount of T cells with TCR$\gamma\delta$ type receptor this reliability check may exceed the maximum variability [39, 40].

It is well known that the immune system matures with subject's age and the distribution of appropriate cell population also differs with age. Younger patients have

more immature cells than older ones and older patients have more antigen-experienced cells. Error in cell maturation may be the sole reason or one of several causes or symptoms of many immune deficiencies. Therefore it may be useful to determine the relative and/or absolute count of naïve and memory T and or B cell populations. Such cells differ in presence of specific surface structures/antigens. In general antigen inexperienced T cells are defined as CD3+CD45RA+ and antigen-experienced T cells as CD3+CD45RO+, with further differentiation between T helper and suppressor subpopulations. Presence of abnormally high relative counts of mature T cells in infants or very small children may reflect feto-maternal chimerism or presence of oligoclonal T lymphocytes. Such cells shall be defined as CD3+CD4+CD45RO+ or CD3+CD45RO+CD8+.

Depending on the purpose of phenotyping further cell characteristics is possible and may be very useful. For example, identification of truly naïve CD4 cells which are CD62L+CD45RA+ allows discrimination between regeneration of naïve cells from thymopoiesis and true immune reconstitution after hematopoietic stem cell transplantation [41]. Highly expanded pool of CD8 T cells which are HLA-DR+CD38+ has prognostic significance for progression of HIV-related disease [41].

Normal values

The relative and absolute sizes of lymphocyte subsets are particularly dynamic during childhood due to maturation and expansion of the immune system during the first years of life. The absolute numbers of B cells expressing CD19 double immediately after birth and decrease subsequently 6.5-fold from 2 years to adulthood, remaining relatively stable thereafter. CD3+ T cells increase 1.5 fold immediately after birth and decrease 3-fold from 2 years to adulthood mainly due to loss of CD45RA+ cells, with the CD4:CD8 T cell ratio falling also down with age [13]. The frequency of T naïve or activated cells declines during infancy. The relative expression of CD45RA and CD45RO isoforms profoundly differs between infants and adults, with much more CD45RA+, as well as activated CD38+ cells and much less antigen-experienced cells in infancy than in adult age. This variation is caused by changes in CD45RA pool due to their release from the thymus. This pattern is, in general, also true for B cells and reflected by functional efficiency of the cells [12, 13].

NK cells comprise approximately 10% of normal lymphocytes in peripheral blood, with their absolute number decreasing almost three-fold within the first 2 months of life and remaining stable thereafter [11, 14]. This is the reason why relative, but also absolute cell counts shall be reported, either calculated from automated complete blood count with differential (dual platform technology) or from the calculation done when standard beads where used (single platform technology) and compared to age-related normal values [12-14].

Protocol selection

Depending on clinical phenotype children who may suffer from primary immunodeficiency may be divided into several categories. Patients with recurrent upper or lower respiratory tract infections may have an antibody defect or complement deficiency. Failure to thrive, intractable diarrhea, opportunistic infections are typical for T-lymphocyte or combined immune deficiencies. Pyogenic bacteria or fungi are frequent pathogens in patients with granulocyte and/or monocyte function deficiencies. Recurrence of infections with particular pathogen may also bring in mind the possibility of diagnosing primary or secondary deficiency. The European Society for Immunodeficiencies (ESID) developed several criteria being a diagnostic guideline for most frequently occurring deficiencies. They provide common definitions for definitive, probable or possible diagnosis [42]. Following this pattern several practical guidelines, taking into consideration an increasing role of flow cytometry in the diagnostics, have been developed [43-46], helping to choose the most efficient protocol for establishing the diagnosis in particular patient.

Rule of first severe, life threatening defects

Diagnostics by flow cytometry is a multistep process that relies on multiparameter analysis with an informative panel of antibodies. In case of potential malignancy the purpose of phenotyping is to discriminate normal from abnormal cells based on presence and absence of various antigens. This is however not the case in diagnostics of immunodeficiencies where the aim is to detect mainly the absence of essential cell populations and functional abnormalities of the remaining ones. Having in mind that primary immune deficiency may be a life threatening defect the common approach to rule out its possibility has been accepted. For this purpose wide screening tests shall be applied.

Severe combined immune deficiencies are a group of inherited disorders that demonstrate abnormalities of T, B and NK cell functions. Such defect is reflected with complete inability to respond to foreign antigens and therefore is potentially life-threatening. Any laboratory using flow cytometry can search for T, B, NK cells, T helper and T suppressor lymphocytes. Depending on results of basic immunophenotyping further tests can be performed. A simple algorithm based on presence or absence of B and NK cells in patients with very low or absent T cells helps in selection of further test (table 1). Flow cytometry can help in verification of suspected common gamma chain (CD132) deficiency or α chain of IL-7 receptor (CD127 deficiency), respectively – in healthy subjects more than 50% of normal peripheral blood lymphocytes shall be positively stained, although it shall be remembered that in some cases results may be false positive (as low CD127 expression in several viral infections) or false negative (in feto-maternal chimerism or in case of reverse mutations).

Table 1. Basic algorithm for diagnosis of life threatening defects [8]

T cells	B cells	NK cells	Suspected primary immune deficiency
negative	positive	positive	IL7RA deficiency CD3ε deficiency
		negative	JAK3 deficiency IL2RA deficiency PNP deficiency
	negative	positive	RAG1/RAG2 deficiency ADA deficiency
		negative	ADA deficiency reticular dysgenesis
positive	positive	positive	MHC deficiency

Major Histocompatibility Complex class II deficiency, originally called bare lymphocyte syndrome, is a rare form of severe combined immunodeficiency with normal numbers of T and B lymphocytes and reduced number of CD4+ T lymphocytes, inherited in autosomal recessive fashion. By flow cytometry it can be detected by absence of MHC class II molecules normally expressed on antigen-presenting cells, such as B lymphocytes or cells from monocyte/macrophage lineage. Very rare Major Histocompatibility Complex Class I deficiency, with milder and more limited phenotype in comparison to bare lymphocyte syndrome, can be detected by absence of surface expression of HLA-class I or β2-microglobulin molecules, despite their presence in the endoplasmic reticulum [47].

Any foreign cells?

Diagnosis of severe combined immune deficiency may be difficult due to relatively frequent phenomenon of maternal cells presence in child's circulation or presences of foreign cells originating from transfusions obscuring the flow cytometric result. Such cells usually demonstrate atypical CD45 expression, with non-Gaussian distribution of fluorescence intensity visible on a histogram (unpublished observation) and frequently unusually high numbers of CD45RO+ T cells either originating from foreign cells or from clonal proliferation of few own child's T cells. In such case determination of CD45RA and CD45RO isoform expression on T cells is very useful, just as determination of T cell receptor (TCRαβ/TCRγδ) clonality.

Defects in production of immunoglobulins

Many defects in immune system may be reflected in aberrant production of immunoglobulins – such defects may remain unnoticed as long as maternal immunoglobulins are present in child's circulation. Determination of immunoglobulin levels belongs to the first steps in evaluation of potentially immune deficient patient. Clinical symptoms and therapeutic options depend on particular molecular defect manifested by complete lack of immunoglobulins, dysgammaglobulinemia or lack of production of specific antibodies.

It is not feasible or cost-effective to sequence all genes potentially involved in pathogenesis of the defect in particular patient. Before molecular diagnostics can be done other techniques shall be applied to detect missing molecules. Flow cytometric analysis in such case begins with determination of relative and absolute B cell counts in peripheral blood. Nobody will be surprised to see a deep defect in immunoglobulin production in patients without B cells. However, depending on the specific defect, treatment options might be different. Patients with SCID due to mutations in RAG, Artemis, BLNK, or CD79a will require hematopoietic stem cell transplantation, but patients with X-linked agammaglobulinemia due to mutation in Bruton tyrosine kinase (Btk) will usually do quite well on substitution therapy. Detection of Btk is possible by Western blot or by flow cytometry [48], although this kind of testing is not widely available due to lack of appropriate equipment as it is in case of Western blot or lack of antibodies working well in flow cytometry. Therefore in patients with agammaglobulinemia and lack of B cells in peripheral blood, bone marrow shall be taken for determination of individual B-cell maturation steps. Any laboratory performing flow cytometry for oncological purposes is also able to evaluate B cell maturation in bone marrow in similar way as it is done in search of B-cell leukemia. This shall be done before performing any molecular testing in search of genetic defect. Knowledge of maturation steps and simple three color staining CD10/CD20/CD19 and TdT/CD10/CD20 will give some clue to where the defect has taken place and which genes shall be sequenced (table 2) [49, 50]. Better discrimination is offered by TdT/CD10/CD20 staining, with most immature precursors being TdT+CD10+ and mature B cell population defined as CD10–CD20+. Pro-B cells, which are CD22+CD19–CD34+, compose a very small population [49, 50]. Early defects will then require sequencing of potentially deficient genes.

Table 2. Particular B cell maturation stages are characterized by expression of various intracellular and surface markers. Stages 1-5 are present in normal bone marrow. Block between stages 1 and 2 are usually caused by mutations in RAG1 or RAG2 and may occur also in Omenn's syndrome. Block between stages 2 and 3 may be caused by Cμ deficiency. XLA is caused by block occurring between stages 3 and 4. Hyper-IgM is manifested by presence of mature cells unable to produce immunoglobulins other than IgM (block between 4 and 5)

1. Pro-B	2. Pre-B-I	3. Pre-B-II	4. Immature B	5. Mature B	6. Plasma cell
CD34	CD34				
TdT	TdT	(TdT)			
	CD10 high	CD10	CD10 low		
	CD19	CD19	CD19	CD19	CD19
		CD20 low	CD20	CD20	
CD22	CD22	CD22	CD22	CD22 high	
	CD45 low	CD45	CD45 high	CD45 high	CD45
		cyIgμ	cyIgμ		cIg
			sIgM	sIgM	

Various types of hyper-IgM syndrome which will require further testing belong to other deficiencies manifested by aberrant immunoglobulin production. Determination of CD40 expression defective in hyper-IgM type III does not require any special procedures, in contrast to CD40 ligand expression which requires T cell stimulation before CD154 molecule is detected on CD4+ T cells. An elegant and fast method for detection of CD40L expression in whole blood was described by M. O'Gorman et al. in 1997 [51]. Abnormalities in CD40 ligand expression are also observed in patients with common variable immunodeficiency syndrome and in HIV infected children, who have decreased T CD4+ cell levels [52].

Common variable immune deficiency belongs to most frequently diagnosed primary immune deficiencies in adult patients. This is a heterogenous group of antibody deficiencies with hard to establish precise molecular defect, but clearly characterized by lack of ability to switch production of immunoglobulins to more mature than IgM and IgD classes. There were several attempts in the past, but Freiburg classification developed by Warnatz et al. appears to bring quite good description of this defect. It refers to patients with more than 1% of B cells in peripheral blood and it is based on differences in frequencies of class-switched memory B cells defined as CD27+IgM–IgD– and immature CD19+CD21– B cells (table 3) [53].

Table 3. Freiburg classification of common variable immunodeficiency using flow cytometry (in all cases B cell comprise more than 1% of peripheral blood lymphocytes (PBL))

Group B cells in PB >1%	Class-witched memory B cells (CD27+IgM–IgD–) (% of PBL)	Immature B cells (CD19+CD21–) (% of B cells)
Ia	<0.4%	>20%
Ib	<0.4%	<20%
II	>0.4%	not applicable

Other defects

Flow cytometry is useful also in detection of several other primary immune defects. A selection of them is presented in table 4. It shall be remembered however that there are several factors affecting the results, among them medication, transfusions, infections, etc. Antibiotics (e.g. cephalosporins) cause increased cellular autofluorescence, corticosteroids decrease CD4+ T cell levels, strenuous exercise decreases lymphocyte counts, and diurnal variation is reflected in changes in absolute lymphocyte values [16].Therefore in any case results may lead rather to probable than to definitive diagnosis.

Final comments

Flow cytometry has become a very useful tool in diagnosis and management of patients suspected to have primary immune deficiency. Determination of numbers of basic lymphocyte subpopulations such as T, B, NK, T-helper and T suppressor cells may be done in many hospital laboratories, as the technique has become extremely popular, leaving more detailed testing to specialized laboratories. Such preliminary results together with general clinical picture may determine further immunological workup, limiting molecular analyses to selected, potentially defective genes. With time, new antigens and availability of antibodies, the number of flow cytometric assays will certainly increase making the diagnostic process faster and more simple, but the technical

Table 4. Useful diagnostic parameters in flow cytometry in various primary immune deficiencies [8, 46, 54]

Primary immune deficiency	Lymphocytes					Monocytes	Granulocytes	Flow cytometric marker
	T lymphocytes (CD3+)							
	total CD3+	T helper CD3+CD4+	T suppressor/ cytotoxic CD3+CD8+	B CD19+ or CD20+	NK CD3– CD16+/CD56+			
severe combined immune deficiencies								
common gamma chain (common γ chain)	↓	↓	↓	N	↓			CD132
JAK3 deficiency	↓	↓	↓	N	↓			
IL2Rα deficiency	↓	↓	↓	N	↓			CD25
CD45 deficiency	↓	↓	↓	N	↓			CD45
IL7Rα deficiency	↓	↓	↓	N	N			CD127
CD3δ deficiency	↓	↓	↓	N	N			
recombination activating genes 1 and 2 deficiency (RAG1/RAG2)	↓	↓	↓	↓	N			
Omenn's syndrome (partial RAG deficiency)	↓	↓	↓	↓	N			oligoclonal T lymphocytes
adenosine deaminase deficiency (ADA)	↓	↓	↓	↓	↓			
bare lymphocyte syndrome (MHC II)	N	↓	N	N	N			MHC class II on B cells/monocytes
ZAP70 protein deficiency	N	N	↓	N	N			
MHC class I deficiency	N	N	↓	N	N			surface MHC I
humoral /antibody deficiencies								
Bruton agammaglobulinemia (XLA)	N	N	N	↓	N			Btk on monocytes
common variable immune deficiencies	↓ or N	N	N					IgD, IgM, CD21, CD27 on B lymphocytes
X-linked HIM	N	N	N	N	N			CD154 on activated CD4+ T cells

Table 4. Continue

Primary immune deficiency	Lymphocytes							
	T lymphocytes (CD3+)							
	total CD3+	T helper CD3+CD4+	T suppressor/ cytotoxic CD3+CD8+	B CD19+ or CD20+	NK CD3– CD16+/CD56+	Monocytes	Granulocytes	Flow cytometric marker
other well defined defects								
Wiskott-Aldrich syndrome	↓ or N			N	N			WASP in lymphocytes absent or decreased CD43 on lymphocytes or monocytes
DiGeorge syndrome (CATCH22)	↓ or N		N	N				
autoimmune lymphoproliferative syndrome (ALPS)	N			↑	N			reduced/absent CD95 elevated CD4-CD8-TCRαβ+ elevated CD19+CD5+ reduced CD4+CD25+CD3+
chronic granulomatous disease (CGD)	N			N	N		reduced/absent flavocytochrome b558 on neutrophils/monocytes in some patients; reduced oxidative burst in some patients	
MPO deficiency						MPO deficient		
leukocyte adhesion deficiency-1 (LAD-I)	N			N	N	absence of CD11/CD18 on monocytes		
leukocyte adhesion deficiency-2 (LAD-II)	N			N	N		absence of CD15 on neutrophils	
interferon gamma receptor deficiency (CD119)						reduced or absent CD119		
interleukin 12 receptor deficiency (CD212)								reduced or absent CD212 on CD3+CD25+ cells

↓ – *reduced;* ↑ – *increased; N – normal.*

principles described above will probably still have to be observed.

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