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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Diagnostic significance of immunophenotyping of peripheral blood lymphocytes in pediatric patients from the Autonomous Province of Vojvodina, Republic of Serbia

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SUMMARY

Introduction/Objective Although lymphocyte immunophenotyping based on flow cytometry is a powerful tool in the diagnosis of many primary immunodeficiences (PID), there has been an increasing awareness of associated costs and the need for its reassessment as a screening tool.
We present the results and diagnostic impact of immunophenotyping performed by flow cytometry in the University Children's Hospital, Belgrade, in a series of patients referred from the Institute for Child and Youth Health Care of Vojvodina from July 2008 to July 2018.

Methods We reviewed the laboratory reports on numbers of B lymphocytes (CD19+), T lymphocytes (CD3+), natural killer cells (CD3–CD16/CD56+) and activated T cells (CD3+HLA-DR+), as well as CD4+ and CD8+ T cells in 198 children.

Results Patients were grouped by stated indication into the following eight categories: hypogammaglobulinemia (34), selective IgA deficiency and/or IgG subclass deficiency (43), various infections with no immunoglobulin deficiencies (67), asthma and/or allergies with no immunoglobulin deficiencies or infections (23), known or suspected autoimmune disorders (24), and miscellaneous diagnoses not accompanied by infections (7). In total, 159 (80.3%) findings were either completely within the respective reference range or exhibited only minimal aberrations. Four patients were diagnosed with Bruton's disease and one with Artemis immunodeficiency. Nineteen patients were given immunoglobulin substitution to control infections and/or maintain immunoglobulin G levels.

Conclusion Lymphocyte immunophenotyping aids the diagnosis of PID in selected patients. We venture some thoughts on how the usefulness of this laboratory method could be improved in real-life tertiary care pediatric hospital settings.

Keywords: immunophenotyping; flow cytometry; lymphocytes; immunodeficiency; children

INTRODUCTION

Immunophenotyping of blood lymphocytes by flow cytometry is a valuable diagnostic tool in many primary and some secondary immunodeficiencies. In its basic capacity, this method usually yields information about absolute and relative abundances of main lymphocyte populations: B lymphocytes ($CD19^+$), T lymphocytes ($CD3^+$) and natural killer (NK) cells ($CD3^-CD16/CD56^+$), as well as main T-cell subpopulations ($CD4^+$ helper T cells and $CD8^+$ cytotoxic/suppressor T cells, with calculation of the CD4:CD8 ratio), and the percentage and absolute number of activated T cells ($CD3^+HLA\text{-}DR^+$). A rare, but diagnostically important subpopulation of "double negative" T cells ($CD4^-CD8^-$) can also be detected [1].

Primary immunodeficiency disorders (PID) that can be diagnosed and evaluated in this way include severe combined immunodeficiency, Omenn syndrome, Wiskott–Aldrich syndrome, agammaglobulinemia (X-linked or autosomal recessive), and primary NK cell deficiencies, while in many other PID immunophenotyping can significantly contribute to the establishment of diagnosis or the assessment of disease severity [2]. This is particularly true for 22q11.2 deletion syndrome (Di George syndrome), where enumeration of immune cells is an obligatory part of patient workup [3, 4], and autoimmune lymphoproliferative syndrome (ALPS; [5, 6]. Likewise, in children (as in adults) with hypogammaglobulinemia, selective IgA deficiency or IgG subclass deficiency, most diagnostic protocols require a checkup of absolute number of B cells in order to uncover potential B-cell immunodeficiencies [7, 8]. In addition, many other PID, such as hyper-IgM syndrome, are amenable to diagnosis by flow cytometry using antibodies specific to the molecule with impaired expression [9].

Among secondary immunodeficiencies, by far the most important indication for

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immunophenotyping is the need for enumeration of $CD4^+$ T lymphocytes in human immunodeficiency virus-infected persons [10]. The method is also used in monitoring the effects of immunosuppressive treatment, particularly when rituximab is administered, or planned to be administered [11]. In other secondary immunodeficiency settings, immunophenotyping usually plays only a limited diagnostic role; it is, however, often necessary to perform it in order to rule out (some) PID in cases when the etiology is less than clear [12]. It should be emphasized that, according to most diagnostic protocols and recommendations, immunophenotyping does not constitute a first- (nor even a second-) level laboratory test [13, 14, 15]. Following the appropriate ordering of tests is crucial for both diagnostic efficiency and cost-effectiveness. On the other hand, early diagnosis of PID is imperative, since it is a major determinant of long-term prognosis.

In this paper, we present the general results and diagnostic impact of immunophenotyping performed by flow cytometry in the Laboratory for Immunology, University Children's Hospital, Belgrade, in a series of patients referred by immunologists from the Child and Youth Health Care of Vojvodina.

METHODS

We reviewed the laboratory reports of 198 children aged from two months to 16 years (76 girls and 122 boys) who were referred by a pediatric immunologist from the Institute for Child and Youth Health Care of Vojvodina for immunophenotyping of peripheral blood lymphocytes to the University Children's Hospital in the period from July 2008 to July 2018.

Briefly, immunophenotyping was performed on Beckman Coulter FC500 Flow Cytometer (Beckman Coulter Inc., Brea, CA, USA) using commercial fluorochrome-conjugated monoclonal antibodies (Miltenyi Biotec, Bergisch Gladbach, North Rhine-Westphalia, Germany) with specificity to human molecules CD3, CD4, CD8, CD16, CD19, CD45, CD56 and HLA-DR. Lymphocyte population was gated on the diagram representing the intensity of CD45 expression and side scatter. In this population, percentages of B lymphocytes ($CD19^+$), T lymphocytes ($CD3^+$), NK cells ($CD3^-CD16/CD56^+$) and activated T cells ($CD3^+HLA\text{-}DR^+$) were determined. Absolute numbers of these subpopulations were calculated based on total lymphocyte number determined using an automated cell counter. The CD4/CD8 ratio was also calculated. Measured abundances of all subpopulations were compared to age-specific reference ranges [16]. For the purpose of this study, we separately evaluated deviations from reference values in the following categories: increase in absolute number of T, B, or NK cells, and alterations of the CD4/CD8 ratio not accompanied by abnormal absolute number of $CD4^+$ or $CD8^+$ cells. These were designated as minimal aberrations (MA).

This paper has been approved by the Ethical Committee of the University Children's Hospital in Belgrade.

RESULTS

According to stated indications for analysis, all patients can be grouped into following eight categories: hypogammaglobulinemia (34), selective IgA deficiency and/or IgG subclass deficiency (43), various infections with no immunoglobulin deficiencies (67) asthma and/or allergies with no immunoglobulin deficiencies or infections (23), known or suspected autoimmune disorders (24), and miscellaneous diagnoses not accompanied by infections (7). The miscellaneous category was comprised of one child with short bowel syndrome after ileostomy performed after repeated episodes of gastroenterocolitis of unknown etiology in infancy, one with ill-defined neutrophil defects and a developmental disorder, one with ataxia, one with unexplained lymphocytosis, one with fever of unknown origin and one whose records, including reasons for referral, have been lost.

All investigated populations were found to be in their reference ranges in 11 (32.4%) of patients with hypogammaglobulinemia; 23 (53.5%) of patients with selective IgA deficiency and/or IgG subclass deficiency; 32 (47.8%) of patients with infection without immunoglobulin deficiencies; 14 (60.9%) of patients with asthma and/or allergies with no immunoglobulin deficiencies or infections, 11 (45.8%) of patients with autoimmune disorders and four (57.1%) patients in the miscellaneous group, for a total of 95 (48.0%) completely normal findings overall.

However, when we add to normal findings those in the MA category, as defined above, the numbers of patients with unremarkable immunophenotype were as follows: 26 (76.5%) of patients with hypogammaglobulinemia; 40 (93.0%) of patients with selective IgA deficiency and/or IgG subclass deficiency; 51 (76.1%) of patients with infection without immunoglobulin deficiencies; 17 (73.9%) of patients with asthma and/or allergies with no immunoglobulin deficiencies or infections, 19 (79.2%) of patients with autoimmune disorders and six (85.8%) patients in the miscellaneous group, for a total of 159 (80.3%) findings that are either completely within the respective reference range or exhibit only MA.

By year of analysis, the proportion of normal findings was 2/5 (40%) in 2008, 3/5 (60%) in 2009, 2/5 (40%) in 2010, 2/12 (16.7%) in 2011, 2/8 (25%) in 2012, 1/9 (11.1%) in 2013, 6/11 (54.5%) in 2014, 16/28 (57.1%) in 2015, 23/46 (50%) in 2016, 35/43 (81.4%) in 2017, and 10/26 (38.4%) in 2018.

B-cell defects

A reduced absolute number of B cells ($CD19^+$) was found in eight patients (23.5%) with hypogammaglobulinemia (five of whom, or 14.7% of all hypogammaglobulinemia patients, had severely decreased number of B cells, defined as < 2% of total lymphocytes); two (4.6%) patients with selective IgA deficiency and/or IgG subclass deficiency; eight (11.9%) patients with infections, two of whom (3%) had a severe decrease (with one of those two showing prompt recovery of B-cell number, returning to normal

range on follow-up examination two months later); three (13%) patients with asthma/allergies; six (25%) patients with autoimmune disorders, one of whom with pancytopenia (4.1%) had a severe decrease exhibiting a reduction of all lymphocyte subpopulations, with recovery on subsequent investigations (although the absolute B-cell number was rather slow to normalize, remaining somewhat below the reference range after two months); and none of the patients in the miscellaneous group. An increase in the absolute number of B lymphocytes, regarded as MA in this study, was noted in two patients (5.9%) with hypogammaglobulinemia, one patient (2.3%) from the selective IgA deficiency/IgG subclass deficiency group, two patients (3%) with infections, and one patient (4.2%) in the autoimmunity group.

Among the patients immunophenotyped for hypogammaglobulinemia, four were genetically diagnosed with a hereditary B-cell defect (Bruton's disease), while one turned out to have a combined (Artemis) deficiency. All of those five patients had relative B cell numbers below 2%.

T-cell defects

The absolute number of T cells ($CD3^+$) was found to be below the lower boundary of the age-appropriate reference range in one patient with hypogammaglobulinemia (2.9%), one patient in the infections group (1.5%), two patients with autoimmunity (4.2%) and one patient in the miscellaneous group (14.3%). On the other hand, an increase of the absolute number of T cells above the reference range (MA finding) was noted in two patients with hypogammaglobulinemia (5.6%), three patients with selective IgA deficiency and/or IgG subclass deficiency (7%), one patient with allergies/asthma (4.3%), 10 patients with infections (14.9%), one patient with autoimmunity (4.2%), and one patient in the miscellaneous group (14.3%).

The patient with reduction in both T and B lymphocytes was diagnosed with Artemis deficiency, as noted above.

The CD4/CD8 ratio was reduced in four patients with hypogammaglobulinemia (11.8%), two patients with selective IgA deficiency and/or IgG subclass deficiency (4.7%), two patients with allergies/asthma (8.7%), 12 patients with infections (17.9%), and four patients with autoimmunity (16.7%). Of these, the absolute number of $CD4^+$ T cells was reduced in just one patient from the infections' group and one from the autoimmunity group. Conversely, the CD4/CD8 ratio was found to be increased in two patients with hypogammaglobulinemia (5.6%), three (6.8%) patients with selective IgA deficiency and/or IgG subclass deficiency (7%), three (13%) patients with allergies/asthma, two patients with infection, two patients with autoimmune phenomena (8.3%), and one patient classified as miscellaneous (14.3%). In all of the above instances except one patient with hypogammaglobulinemia, increased CD4/CD8 ratio was not accompanied with a reduction of the absolute number of $CD8^+$ T cells below the reference range, and were therefore regarded as MA.

An increased number of activated T lymphocytes ($CD3^+HLA\text{-}DR^+$) was noted in one patient with hypogammaglobulinemia (2.9%), one patient with selective IgA deficiency and/or IgG subclass deficiency (2.3%), six patients with infections (9.0%), and two patients with autoimmune disorders (8.3%).

Natural killer cell deficiency

A reduction in the absolute number of NK cells was found in one patient (2.9%) in the hypogammaglobulinemia group, two (8.7%) of patients in the asthma/allergy group, five (7.5%) in the infections group, and three (12.5%) in the autoimmunity group.

Hypogammaglobulinemia

As noted above, out of 34 patients in this category, eight (23.5%) had a reduced number of B cells, including five (14.7%) with severe reduction. The remaining 26 (76.5%) patients had normal or MA findings. Apart from five patients with genetically confirmed primary immunodeficiencies, who were all in this group, five more patients received immunoglobulin substitution, one of whom had a moderately decreased B-cell count. Thus, immunoglobulins were received by 10 (29.4%) children with hypogammaglobulinemia overall.

Selective IgA/IgG subclass deficiency

In this group, as noted above, two (4.6%) patients had a reduction of absolute number of B cells, while one (2.3%) had an increased number of activated T lymphocytes. The remaining 40 (93%) patients had either normal or MA immunophenotype. Substitution therapy was introduced by attending immunologist in 13 patients in this group: one patient with selective IgA deficiency solely and a B-cell count decrease, three patients with both IgA and IgG subclass deficiency and nine with IgG subclass deficiency alone, all of whom had normal B-cell counts.

Asthma/allergy with no immunoglobulin deficiencies or infections

Three (13%) patients in this group demonstrated reduced B-cell numbers, one of whom also had a reduction of NK cells. Another patient (4.3%) had an isolated reduction of NK cells. In addition, two (8.7%) patients had an increased number of activated T cells. The remaining 17 (73.9%) patients had normal or MA findings. Out of 23 patients with isolated asthma/allergy, none received immunoglobulin substitution.

Asthma/allergy combined with immunoglobulin deficiencies, infection or autoimmunity

In addition to the 23 patients with isolated asthma/allergy, 15 patients had asthma or allergy combined with hypogammaglobulinemia. Of these, 13 (86.7%) had normal findings or MA, one had a significant reduction of B-cell number (diagnosed as Bruton's disease), and one

had a combined reduction of B and T-cell numbers with subsequent recovery. Another 15 patients had a combination of asthma/allergy and selective IgA deficiency, with 14 (93.3%) exhibiting normal/MA immunophenotype and the remaining one a modest reduction of B cells. Thirteen children had a combination of asthma/allergy with IgG subclass deficiency. In this group, all findings were either normal or MA. Eighteen patients had a combination of asthma/allergy and infection without immunoglobulin abnormalities, twelve of whom had normal and three MA findings (83.3%). Two patients had increased number of activated T lymphocytes, one had moderate reduction in both B and NK cells. All six patients whose asthma/allergy was combined with autoimmunity had findings within the reference range.

Infections with no immunoglobulin deficiencies

In this group, eight (11.9%) patients had a decreased number of B cells, (with one of those two showing recovery of B-cell number, returning to normal range on follow-up examination two months later) and another patient is lost for follow-up. Three of eight patients with low number of B cells also had a low number of NK cells. Low number of NK cells was found in two additional patients (one who also had a reduction of $CD4^+$ T cells and the other with an isolated reduction of NK cells). Thus, in total, 5 (7.5%) patients had a reduced number of NK cells. Six patients in this group displayed an increased number of activated T cells. The total number of patients in this group who had normal or MA findings was 51 (76.1%). One patient with asthma and bronchiectasis in this category received immunoglobulin substitution, with all investigated lymphocyte populations within reference values.

Autoimmune diseases

Of the 24 patients with known or suspected autoimmune diseases, 12 (50%) had connective tissue disorders. Eleven of these (91.7%) had normal findings or MA, while one showed an increased number of activated T cells. Five patients (20.8%) had inflammatory bowel disease, all with normal/MA findings. Three (1.2%) were evaluated because of cytopenias; two of them, with thrombocytopenia, had a normal immunophenotype, while the third child, who had pancytopenia, exhibited a reduction of all lymphocyte subpopulations attributable to the pancytopenia itself, with recovery on subsequent investigations (although the absolute B-cell number was rather slow to normalize, remaining somewhat below the reference range after two months). The remaining four children were subjected to immunological examination for various reasons: two for lymphadenitis, one accompanied by *erythema multiforme*, a combination of glomerulonephritis and Hashimoto thyroiditis, and isolated splenomegaly, respectively. They all had normal findings or MA. None of these patients received immunoglobulin substitution.

DISCUSSION

Although lymphocyte immunophenotyping based on flow cytometry is a powerful tool in the diagnosis of many primary immunodeficiencies, there has been an increasing awareness of associated costs and the need for its reassessment as a screening tool in the diagnosis of PID [17].

The results we present here constitute referrals by the only tertiary center for immunodeficiencies for peripheral blood lymphocyte immunophenotyping to the University Children's Hospital in Belgrade. Pediatric population of the Autonomous Province of Vojvodina counts around 300,000 children, but we cannot exclude that patients from Vojvodina were refereed to another tertiary care pediatric institution in Belgrade directly, without being seen by immunologists in Novi Sad. The exact prevalence of primary immunodeficiencies in Serbia (or Vojvodina) is unknown at this time. Using the data from the German National Registry of Primary Immunodeficiencies (PID-NET), where the minimal prevalence of PID is stated to be 2.72 per 100,000 inhabitants [18] we should expect 45–50 people with PID living in Vojvodina (approximately 1.7 million inhabitants). We actually found five patients with genetically confirmed immunodeficiency and additional 19 patients requiring therapy for immunodeficiencies during a ten-year period. In our patients, lymphocyte subpopulation analysis was practically performed as an initial test, together with immunoglobulin levels. As shown by our results, this approach yielded a relatively low proportion of findings of diagnostic importance. All patients except one that were diagnosed with PID or received therapy suffered from either hypogammaglobulinemia or IgG subclass deficiency. Nineteen patients were given immunoglobulin substitution in order to control infections or/and to maintain immunoglobulin G levels without confirmed immunodeficiencies, an approach used by other authors as well [19]. A moderately decreased B cell count was found in only one patient in this group.

The rationale for our decision to subsume non-specific findings under the category of MA was that these particular lymphocyte abnormalities are not, by itself, diagnostic or strongly indicative of any particular PID according to the guidelines of the European Society for Immunodeficiencies [20]. In our experience, such findings appear to be of quite limited clinical value, and thus we considered the sum of patients with this type of findings and those with all findings within the normal range informative regarding the diagnostic value of the analysis. Thus only ~20% of analyses in this series resulted in "positive" findings. Even among the latter, we noted that isolated aberrations of NK cells also appear to be of limited value. We have to emphasize that we did not have patients with severe combined or other complex immunodeficiencies where enumeration of NK cells might be of diagnostic significance. NK cell deficiency-associated PID are quite rare, and none of our patients with a low absolute number of NK cells exhibited the clinical signs of respective disorders (as stated in the European Society for Immunodeficiencies guidelines), such as *GATA2* deficiency, accompanied by

susceptibility to mycobacteria, papillomaviruses, histoplasmosis and lymphedema [20]. Furthermore, the number of activated T cells ($CD3^+HLA\text{-}DR^+$) usually reflects ephemeral changes related to some current infection or other factors. This might justify their inclusion in the MA category, although T-cell activation status could be useful as part of an extended immunophenotype, particularly in patients with autoimmune disorders. Finally, reduction in absolute numbers of $CD4^+$ or $CD8^+$ T cells was also rare in our patients, and not particularly informative. We are therefore inclined to agree with Dias et al. [17] that the enumeration of the above subpopulations can, as a screening test, hardly be cost-effective on a large scale.

Furthermore, if we analyze the proportion of normal findings by year of analysis, we did not see an increase in "positive" findings, indicating that there was no change in referral policy. However, here we must add a *caveat* that in some cases (e.g., lymphopenia in the first months of life, early-onset inflammatory bowel disease, or clinical suspicion of a severe combined immunodeficiency) a normal immunophenotype can be diagnostically important. We would like to highlight the need for more precise guidelines and standardized indications for testing, as well as for improving the communication between clinicians who order tests and specialists who perform and evaluate them. On the other hand, for the diagnosis of specific PID, a more detailed immunophenotypic analysis is necessary: one that would include further B- and T-cell subpopulations (such as naïve and memory cells) or subtypes (e.g., Th1, Th2, Th17, Treg). Flow cytometry could also be helpful in the investigation of immune cell function (oxidative burst, cytotoxicity), cytokine production, mitogen- or antigen-induced cell proliferation, signaling pathways, or specific protein expression pertinent to the diagnosis of PID. Such tests are planned to be introduced in our center in the near future, highlighting the need to ensure that they will be used in accordance with proper indications, supported by relevant European Society for Immunodeficiencies or other guidelines.

CONCLUSION

Lymphocyte immunophenotyping can contribute to the diagnosis of PID in selected patients. However, the usefulness of this laboratory method in real-life tertiary care pediatric hospital settings could be significantly improved by strict adherence to indications and further integration towards a comprehensive diagnostic approach.

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Дијагностички значај имунофенотипизације лимфоцита периферне крви код педијатријских болесника из Аутономне Покрајине Војводине, Република Србија

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САЖЕТАК

Увод/Циљ Мада је имунофенотипизација лимфоцита заснована на проточној цитометрији моћно оруђе у дијагностици многих примарних имунодефицијенција, постоји нарастајућа свест о трошковима које ова метода повлачи и потреба за преиспитивањем њене улоге у скринингу на поменута обољења. Приказујемо резултате и дијагностички значај имунофенотипизације помоћу проточне цитометрије изведене у Имунолошкој лабораторији Универзитетске дечје клинике у Београду код серије болесника упућених из Института за здравствену заштиту деце и омладине Војводине у периоду од јула 2008. до јула 2018.

Методе Анализирали смо лабораторијске налазе броја Б-лимфоцита (*CD*19+), Т-лимфоцита (*CD*3+), урођено убилачких ћелија (*CD*3–*CD*16/*CD*56+) и активираних Т-ћелија (*CD*3+*HLA-DR*+), као и *CD*4+ и *CD*8+ Т-ћелија код 198 деце.

Резултати Болесници су груписани према назначеним индикацијама у следећих осам категорија: хипогамаглобулинемија (34), селективна *IgA* дефицијенција и/или дефицит поткласе *IgG* (43), разне инфекције без имуноглобулинских дефицијенција (67), астма и/или алергије без имуноглобулинских дефицијенција или инфекција (23), потврђене или суспектне аутоимунске болести (24) и разне дијагнозе које нису биле праћене инфекцијом (7). Укупно 159 (80,3%) налаза је у целини било у одговарајућем референтном опсегу или је показивало тек минимална одступања. Код четири болесника је постављена дијагноза Брутонове болести, а код једног је откривена имунодефицијенција Артемис. Деветнаест болесника је примало имуноглобулинску супституцију ради сузбијања инфекција и/или одржавања нивоа *IgG*.

Закључак Имунофенотипизација лимфоцита доприноси дијагностици примарних имунодефицијенција код одабраних болесника. Прилажемо одређена размишљања о томе како би се учинак ове лабораторијске методе у реалним условима терцијарних педијатријских установа могао побољшати.

Кључне речи: имунофенотипизација; проточна цитометрија; лимфоцити; имунодефицијенција; деца