Utility of leucocyte antigens in distinguishing between bacterial and viral infection in children

ANNA STELMASZCZYK-EMMEL[1], ANNA SOSNOWSKA[2], JUSTYNA KURKOWIAK[3], MAGDALENA SAGAŁA[4], LIDIA ZAWADZKA-GŁOS[5], BEATA PYRŻAK[4], URSZULA DEMKOW[1]

[1]Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, Medical University of Warsaw, Warsaw, Poland
[2]Student's Scientific Group at the Department of Laboratory Diagnostics and Clinical Immunology of Developmental Age, Medical University of Warsaw, Warsaw, Poland
[3]Department of Physical Chemistry, Medical University of Warsaw, Warsaw, Poland
[4]Department of Paediatrics and Endocrinology, Medical University of Warsaw, Warsaw, Poland
[5]Department of Paediatric Otolaryngology, Medical University of Warsaw, Warsaw, Poland

Abstract

Introduction: *Accurate diagnosis of bacterial and viral infection is very difficult. Unfortunately, there is still no quick and discriminative diagnostic test that would help clinicians in establishing the diagnosis and taking a decision on treatment. The aim of the study was to compare the expression of antigens on phagocytes, which are involved in the first defence line during bacterial and viral infections in children, as a potential tool to distinguish the etiology of the infection.*

Material and methods: *The expression of CD35, CD32, CD88, and MHC class I on phagocytes in 49 blood samples from children with high fever and suspected infection as well as 19 healthy children (control group) was assessed by flow cytometry. Thirty-three children were diagnosed with bacterial and 16 with viral infection. Expression of antigens was analysed on a FACSCanto II flow cytometer according to mean fluorescence intensity (MFI) and antibody binding cites (ABC).*

Results: *Significant differences were observed for the following: CD32, CD35, CD88, and MHCI on granulocytes; CD32, CD35, CD88 on monocytes; and MHC-I ratio between groups were observed. The obtained results did not allow us to establish valuable score points for distinguishing between bacterial and viral infections. Classification and a regression tree using CD88 expression on granulocytes and CRP was developed. It enabled us to differentiate between the origin of infection with sensitivity and specificity of more than 90%.*

Conclusions: *Utility of use of wide range antigens' expression on phagocytes for distinguishing between bacterial and viral infection in children has limited value. More adequate seems to be use of CD88 expression on granulocytes linked with CRP value.*

Key words: *CD35, CD88, children, flow cytometry, infection.*

(Centr Eur J Immunol 2018; 43 (3): 262-269)

Introduction

Differential diagnosis of bacterial and viral infection is often a big challenge. The clinical symptoms are so similar or even the same that in most cases it is impossible for physicians to make a quick and accurate diagnosis without employing laboratory tests. Nevertheless bacterial and viral infections are a big diagnostic difficulty also for laboratories. Routine laboratories offer several tests aiming to discriminate inflammation origin, including: complete blood count with white blood cell (WBC) differentiation count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), procalcitonin, serum cytokines levels, but all of them have their disadvantages and limitations. However, they can be useful in establishing the etiology of the infection only in specific cases (e.g. procalcitonin in sepsis), but generally they are not sensitive and specific enough to make an unambiguous decision [1-4]. On the other hand, tests which are known as a gold standard for confirmation of bacterial or viral etiology of infection (microbiological culture or polymerase chain reaction – PCR, respectively) are laborious and, what is more important, time-consuming. Timing of treatment implementation is

crucial in successful disease eradication. Additionally, they also have some limitations, i.e. cultures may give false negative results if the patient already got antibiotics, while PCR may lead to false positive results, because of high sensitivity and presence of viral genome in the sample long after the infection [5].

Not sufficiently fast or uncertain diagnosis leads straight to excessive antibiotic usage, because physicians would rather give antibiotics in advance than put the patients at risk. Such unnecessary antibiotic treatment can cause severe side effects like toxicity, allergic reactions, suppression of immune system, intestinal bacterial flora disruption or even, according to the latest studies, obesity. And what is even more dangerous, it may lead to development of antibiotic resistance. Those side effects are even more harmful in childhood, when immunological responses are still developing and rapidly changing [6-9].

Recently, some studies concerning the usefulness of immunological markers have been conducted. Researches focused on expression of these antigens on phagocytes, which are involved in the first defence mechanisms, including: antigen presentation, recognition and phagocytosis of IgG and complement opsonised pathogens, and complement activity. Attention was paid to the expression of CD64 and CD35 on neutrophils and monocytes, but the expression of CD32, CD88, CD46, CD55, CD59, MHC class I, CD46, CD10, CD66b, and CD282 was analysed as well. Such tests are performed quickly and easily by flow cytometry, and diagnosis can be made within one hour [10-15].

Nuutila *et al.*, in addition to the analysis of antigen expression (by mean fluorescence intensity – MFI and antibody binding sites – ABC), proposed different methods to interpret the results in adults such as BI-index and bacterial infection score (BIS) method. Following their work, we have selected four antigens to be checked in children: CD35 (complement receptor 1), CD32 (Fcγ receptor II), CD88 (receptor for complement-derived anaphylaxin C5a), and HLA-DR (MHC Class I receptor) [10, 12].

The aim of the study was to simplify the recognition of infection etiology in children by comparing the antigens' expression on phagocytes, which are involved in the first defence line during infections.

Material and methods

The study involved 49 children with high fever and suspected infection admitted to the Department of Paediatrics and Endocrinology (Public Paediatric Teaching Hospital, Medical University of Warsaw) and 19 healthy children (control group). Thirty-three children were diagnosed with bacterial and 16 with viral infections. Table 1 shows the clinical characteristics of the participants of the study.

All patients had characteristic symptoms of infection, including fever of at least 38.5°C. Fifteen bacterial and five

viral infections were microbiologically confirmed, and the etiology of infection is shown in Table 1. The patients without microbiological confirmation of infection were diagnosed by physician on the basis of symptoms, laboratory test, and clinical course of the disease. The blood samples were collected before any treatment. Complete blood count (CBC) test was determined from all samples, CRP was assessed from patients' samples only.

Reference values of WBC and leukocyte populations differ depending on age; therefore, children from different age groups were not analysed together. That is why exact values are not presented but the number of decreased or increased values, which were checked against appropriate reference values.

The study was approved by the Independent Ethics Committee of the Medical University of Warsaw. The parents gave informed consent for the participation in the study. The study was conducted according to the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

For flow cytometric analysis only 100 μl of residue blood collected on EDTA (tube for CBC test) was used. Blood samples were collected at the day of admission, before any treatment has started. 50 μl of peripheral blood collected on EDTA was stained with 5 or 10 μl of monoclonal antibodies (according to the manufacturer's instructions, BD-Pharmingen) in one tube (anti-CD35 FITC, anti-CD88 PE, anti-HLA-A,B,C PE-Cy7, anti-CD32 APC, anti-CD14 APC-Cy7, anti-CD45 V450) (BD Pharmingen). Each tube was incubated for 20 minutes in the dark at room temperature (RT). The fixation and the erythrocytes lysis steps were performed with Uti-Lyse (DAKO) reagent. Briefly, 100 μl of Reagent A (Uti-Lyse, DAKO, fixation step) was added. Tube was incubated for 10 minutes in the dark at RT. Then, 1000 μl of Reagent B (Uti-Lyse, DAKO, erythrocytes lysing step) was added and tube was incubated for another 10 minutes in the dark at RT. There was no washing step in this procedure. The samples were analysed on a FACSCanto II flow cytometer (Becton Dickinson) with FACS Diva v8 software. Expression of antigens was analysed according to MFI and ABC. To establish BIS values and Classification and Regression Tree (CART) absolute ABC was used.

An example of a patient's cytometric result with gating strategy is shown in Figure 1.

Instead of using isotype antibody control fluorescence minus one control for each interesting antigen was applied. For absolute ABC count Quantum Simply Cellular anti-Mouse IgG beads (Bang Laboratories, USA) and QuickCal v 2.3 software were used. Cytometer set-up and tracking beads were used to compensate for instrument-related differences between consecutive days.

Statistical analyses were performed using StatSoft, Inc. (2015) STATISTICA, version 13. All data in the groups were tested for normality using a Shapiro-Wilk test. To

Table 1. Clinical characteristics of children with infection and healthy controls

Number	Children with infection		Healthy controls
	49		**19**
Age (in years, mean ±SD)	2.95 ±2.23		8.40 ±5.25
Gender			
Male	27		9
Female	22		10
Type of infection microbiologically confirmed	bacterial (*n* = 33) *Streptococcus pyogenes* (*n* = 2) *Escherichia coli* (*n* = 6) *Streptococcus pneumoniae* (*n* = 2) *Staphylococcus aureus* (*n* = 2) *Klebsiella oxytoca* (*n* = 1) *Campylobacter jejuni* (*n* = 1) *Mycoplasma pneumoniae* (*n* = 1)	viral (*n* = 16) rotavirus (*n* = 3) RSV (*n* = 1) EBV (*n* = 1)	NA
WBC (cells/µL) (%)			
Below reference value	1 (3)	0 (0)	0 (0)
In reference value	16 (48.5)	11 (68.8)	18 (94.7)
Above reference value	16 (48.5)	5 (31.2)	1 (5.3)
Lymphocytes (cells/µL) (%)			
Below reference value	8 (24.2)	4 (25)	0 (0)
In reference value	25 (75.8)	12 (75)	16 (84.2)
Above reference value	0 (0)	0 (0)	3 (15.8)
Lymphocytes (%)			
Below reference value	31 (93.9)	14 (87.6)	5 (26.3)
In reference value	2 (6.1)	1 (6.2)	9 (47.4)
Above reference value	0 (0)	1 (6.2)	5 (26.3)
Neutrophils (cells/µL) (%)			
Below reference value	0 (0)	1 (6.2)	2 (10.5)
In reference value	12 (36.4)	7 (43.8)	16 (84.2)
Above reference value	21 (63.6)	8 (50)	1 (5.3)
Neutrophils (%)			
Below reference value	0 (0)	1 (6.2)	6 (31.6)
In reference value	4 (12.1)	1 (6.2)	8 (42.1)
Above reference value	29 (87.9)	14 (87.6)	5 (26.3)
CRP (mg/dL) (%)			
< 1	5 (15.2)	5 (31.3)	NA
> 1	28 (84.8)	11 (68.7)	
Average	8.153	1.325	

NA – not applicable

compare the means of two groups, a t-test was applied if the variances of the two populations were equal. If not, a Cochran-Cox test was applied (for comparison of the variance between two groups, an F-test was employed). In the case of lack of normality, the Mann-Whitney test was used.

The statistically significant difference between three groups was checked using one-way ANOVA or the non-parametric equivalent – Kruskal-Wallis test. The assumption of homogeneity of variance was performed with Brown-Forsythe test. For post-hoc analysis Tukey's test was applied.
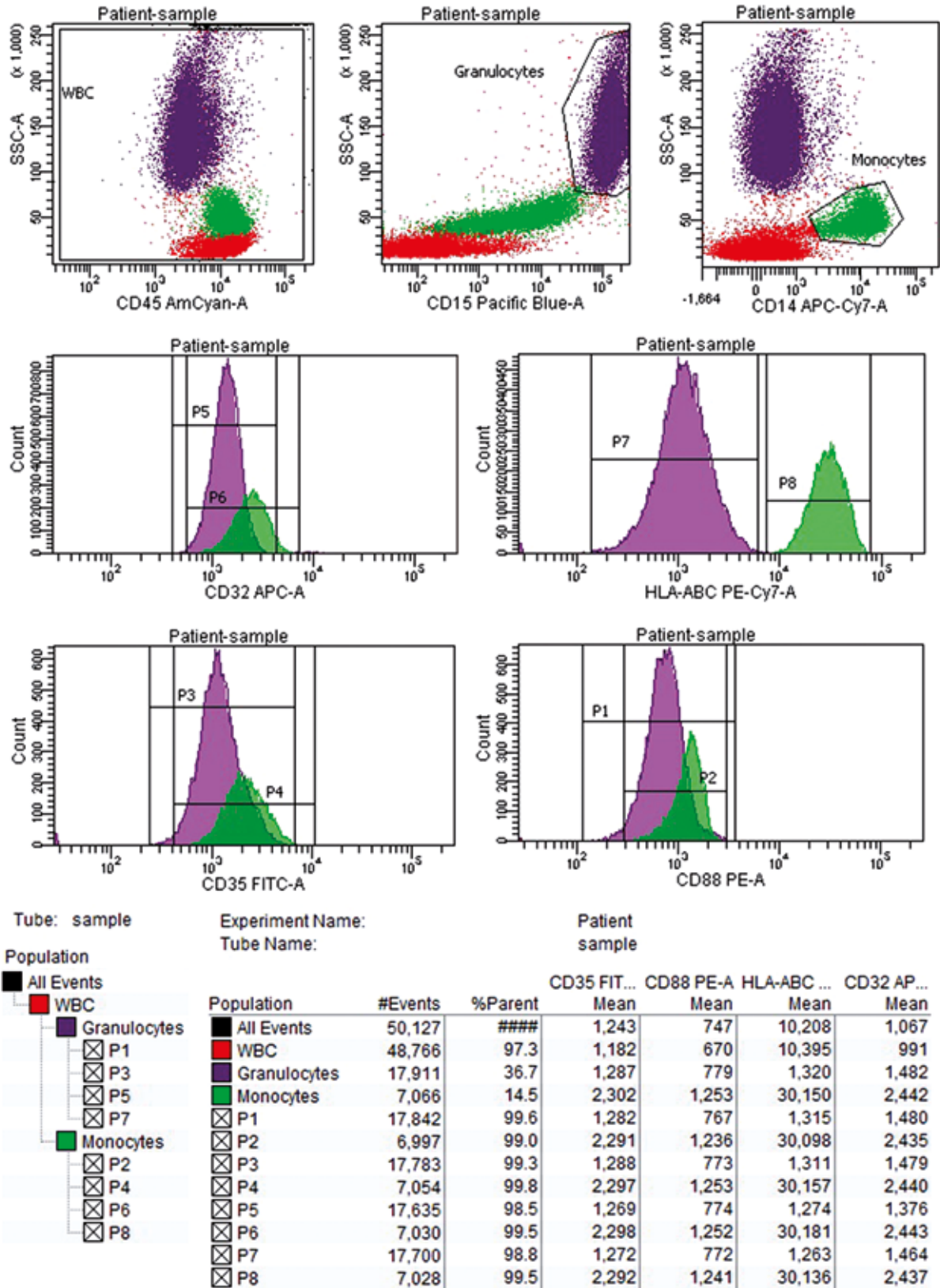
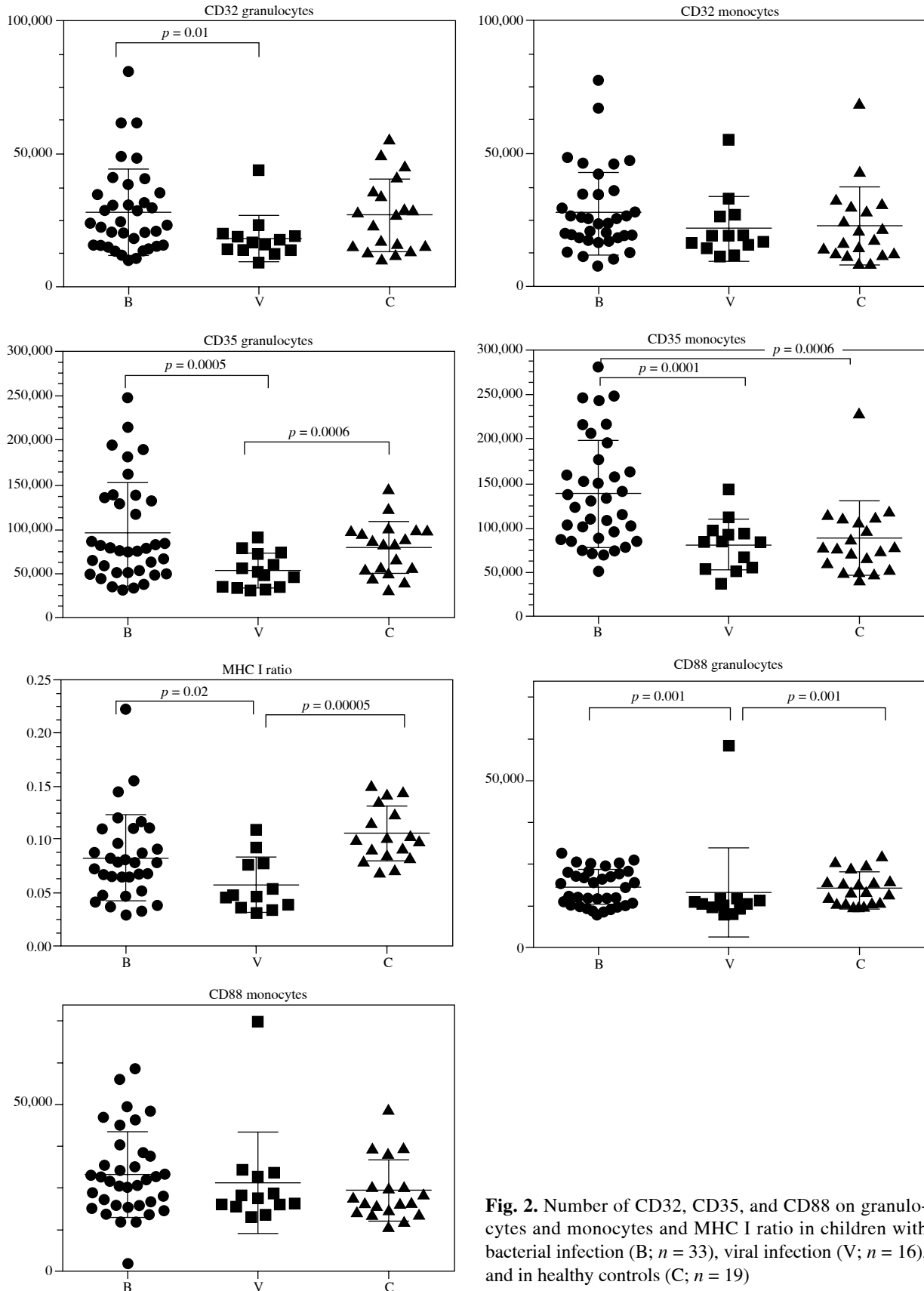**Fig. 1.** The example of patient cytometric result with gating strategy

Fig. 2. Number of CD32, CD35, and CD88 on granulocytes and monocytes and MHC I ratio in children with bacterial infection (B; n = 33), viral infection (V; n = 16), and in healthy controls (C; n = 19)

The efficiency of the BIS variable in differentiating between viral and bacterial infections was evaluated using ROC curve analysis. In determining the optimal cut-off values the tangent method and the Youden index method were applied. To measure the accuracy of the test the area under the ROC curve (AUC) was determined.

To create a predictive model, CART analysis was applied. The purpose of this analysis was to learn how one can discriminate between the two types of infections, based on the tested parameters. Each independent variable was examined, and a split was made to maximise the sensitivity and specificity of the classification, resulting in the development of a decision tree. The Gini Index method was used to split off the largest category into separate groups, and the misclassification costs were taken to be equal for every class.

All computations were applied at the significance level 0.05.

Results

Routine laboratory tests

Among routine laboratory tests analysed in our study (WBC, lymphocytes, neutrophils, CRP – shown in Table 1) only CRP was significantly lower in children with viral infection compared to children with bacterial infection ($p = 0.0017$).

Expression and number of antigens on monocytes and granulocytes

Expression of antigens was directly assessed using MFI. Then, in order to make the results independent from the laboratory, the analyser and the day of analysis MFI was recalculated and shown as ABC, which shows the exact number of antigens on cells. The results of all tested antigen expressions with statistical significance between groups are shown in Figure 2. The MHC I ratio was calculated as the amount of MHC I on granulocytes divided by the on monocytes. Expressions of antigens on granulocytes and monocytes analysed individually were not adequate to use for differentiation between the etiologies of infection.

BIS value according to Nuutila *et al.* [10]

The rapid BIS test method proposed by Nuutila *et al.* [10] was applied to 68 samples of paediatric patients. The BIS value was obtained by summing up individual variable score points for neutrophil CD35, monocyte CD32, monocyte CD88, and MHCI ratio. The variable score points were calculated using four cut-off values (viral median value = cut-off 1, bacterial Q1 value = cut-off 2, bacterial median value = cut-off 3, and bacterial Q3 value = cut-off 4) proposed in [10].

The cut-off value 5 from the above-mentioned paper has no application to the paediatric patients because in all cases the BIS value was below 0. For this group, the optimal cut-off point of –7 for the BIS value was found using the method which minimises the distance between ROC plot and point

(0;1) as well as for the Youden index method (AUC 82.9%). This cut-off allowed us to correctly classify 93.1% of cases of bacterial infections and only 53.3% cases of viral infections.

It should be emphasised that three out of four parameters, which are part of the BIS value, do not differentiate between the two groups of infections if used separately. The variable score point for the MHC class I ratio for the whole group of children with infection takes the value –8 regardless of the type of infection. Moreover, variable score points for Monocyte CD32 and Monocyte CD88 for infected children do not significantly differentiate the groups. For Monocyte CD32 and CD88 almost all patients score 0 (100% for viral infected patients and 93.9% for bacterial infected patients for Monocyte CD32; 93.7% for viral patients and 87.9% for bacterial patients for Monocyte CD88). For this reason, the ROC curve was used to explore the threshold for the application of variable score points for Neutrophil CD35. The results are almost the same (AUC 81.1%) as for the sum of four variable score points. This parameter allows one to correctly classify 93.9% of bacterial cases and only 50.0% of viral cases. Paired sample statistical method was developed to compare those two variables (the BIS value versus the score points for Neutrophil CD35), giving no significant differences.

BIS value according to our calculations

The cut-off values for variable score points proposed in [10] did not fulfil their role in the group of infected children due to fundamentally different ranges of quartiles used to set down the BIS value. For this reason, in the next step we decided to designate a new cut-off value (as in [10]). However, the variable score point for Monocyte 88 was removed from the analysis because the viral median value (cut-off 1) was greater than the bacterial Q1 value (cut-off 2).

To find the optimal threshold point for the new parameter BIS, the point closest to the (0;1) corner in the ROC plane was determined in the group of paediatric patients, giving the value – 5 (AUC 84.7%). This made it possible to classify 100% of bacterial cases and only 46.7% of viral cases. The Youden index method gave the cut-off value 0. For this value 89.7% of cases of bacterial infection and 66.7% of cases of viral infection were classified correctly.

CART method

Due to unsatisfactory results for the BIS parameter, the CART method was used to develop a model that could clas-
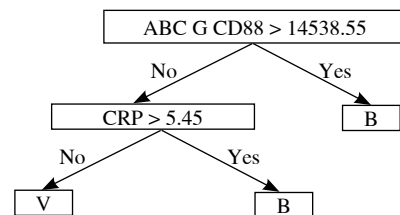

Fig. 3. A decision tree for the infection classification problem

sify patients into two categories: viral and bacterial infections using a set of independent predictor variables. The obtained rule is illustrated in Figure 3.

The above rule allowed us to correctly classify 30 of 33 patients (90.9%) with bacterial infections and 15 of 16 patients (93.75%) with viral infections.

Discussion

During the first years of life the immunological system is not completely formed, and it is changing and developing continuously. The response to an infective agent can be different than in adults [16, 17]. In young age, fast diagnosis of infection etiology and the decision of antibiotic treatment can be crucial because infections may have a fulminant course. On the other hand, unnecessary treatment can result in side effects [9, 18].

Routine laboratory tests are insufficient and rarely give an exact answer. As in our study, although CRP was significantly lower in children with viral than with bacterial infection, this parameter cannot unequivocally discriminate between these two etiologies, as is well known.

That is why, for several years, many authors focused on immunological markers, with enough high specificity and sensitivity in infections to serve as a laboratory parameter suitable to differentiate the etiology of infection. The list of markers that are potentially useful is very long. So far, the most popular and most investigated are CD35 and CD64, CD59, CD55, MHC class I, CD88, CD46, CD10, CD11b, and others [10-15, 19, 20].

All cited authors concordantly indicate that there is up-regulation of CD64 on neutrophils during infection, independently of its origin [13, 15, 19, 21-23]. CD35 is upregulated on neutrophils only during bacterial infection, but specificity and sensitivity of such tests are too low [10, 11, 13, 15, 24]. Also, the combination of these two parameters does not give satisfactory results [13, 15].

Analysing antigen expression alone does not represent sufficiently high specificity and sensitivity, so researchers try to analyse them in many different combinations or create ratios, indexes, and various factors, which enable them to establish reliable parameters with high specificity and sensitivity [10, 12].

On the other hand, the addition of more and more markers make laboratory testing complicated, expensive, and time-consuming, which, in this case, is highly undesirable.

Unfortunately, most researches use only MFI to analyse antigen expression on cells, and therefore there is no possibility to compare the results between different studies, because MFI is specific for one study and one cytometer's parameters. To make such comparison possible, beads for ABC calculation should be used to make the analysis independent from the cytometer setting and day of analysis.

In our study (thanks to ABC beads), we had a possibility to compare the results with analogical studies conducted in adult patients. Also, the study was designed to check if indexes that were promising in adults will be beneficial in children. Nuutila *et al.* proved that in adults (age range 16-85 years old) the results are not age dependent [10]. In our material, several antigens expressed on granulocytes and monocytes in children were definitely different, and as a consequence the application of the BIS created by Nuutila *et al.* was not possible [10]. Based on their calculations, we created our own BIS, but the specificity and sensitivity of such an index was not satisfactory.

Similar studies in paediatric populations are extremely rare. According to our knowledge, only Zhu *et al.* performed such a study [13]. They have shown, similarly to our findings, that antigen expression (CD35 and/or CD64) on neutrophils can serve as an additional parameter to CRP analysis, and such combined test would improve the prediction of the infection etiology. In our study, although the CRP level correlated with CD35 expression on granulocytes, CD88 expression on granulocytes was the best marker for combined analysis with CRP. Zhu *et al.* did not study the antigen densities on cells, so we were not able to directly compare their results with our findings [13].

Taking into consideration all of the analysed antigens (CD32, CD35, CD88, HLA ABC) on granulocytes and monocytes and routine laboratory tests, the Classification and Regression Method for selection of the most valuable parameters was performed. Based on such analysis we have shown that the most useful method to distinguish between bacterial and viral infections in our group of patients is a decision tree based on serum CRP level and CD88 expression on granulocytes. Such analysis is fast, simple, and, in our study with 68 patients, gave satisfactory results (specificity and sensitivity over 90%). The next step is to apply the proposed analysis in a larger group of children and in adults.

Conclusions

Antigen expression obtained from children with infections is definitely different from that obtained from adults. There is no possibility to establish the same parameters or cut-off values appropriate for both adults and children, but the changes of antigen expression go in the same direction.

Utility of use of analysis of a wide range of antigens' expression on phagocytes for distinguishing between bacterial and viral infection in children has limited value. The use of CD88 expression on granulocytes linked with CRP value seems to be more valuable.

The authors declare no conflict of interest.

References

1. Honda T, Uehara T, Matsumoto G, et al. (2016): Neutrophil left shift and white blood cell count as markers of bacterial infection. Clin Chim Acta 457: 46-53.
2. Dubos F, Moulin F, Gajdos V, et al. (2006): Serum procalcitonin and other biologic markers to distinguish between bacterial and aseptic meningitis. J Pediatr 149: 72-76.
3. Chauhan N, Tiwari S, Jain U (2017): Potential biomarkers for effective screening of neonatal sepsis infections: An overview. Microb Pathog 107: 234-242.
4. Dymicka-Piekarska V, Wasiluk A (2015): Procalcitonin (PCT), contemporary indicator of infection and inflammation. Postepy Hig Med Dos 69: 723-728.
5. Fan SL, Miller NS, Lee J, Remick DG (2016): Diagnosing sepsis – The role of laboratory medicine. Clin Chim Acta 460: 203-210.
6. Azad MB, Moossavi S, Owora A, Sepehri S (2017): Early-Life Antibiotic Exposure, Gut Microbiota Development, and Predisposition to Obesity. Intestinal Microbiome: Functional Aspects in Health and Disease 88: 67-79.
7. Azad MB, Bridgman SL, Becker AB, Kozyrskyj AL (2014): Infant antibiotic exposure and the development of childhood overweight and central adiposity. Int J Obes 38: 1290-1298.
8. Principi N, Esposito S (2016): Antibiotic administration and the development of obesity in children. Int J Antimicrob Agents 47: 171-177.
9. Mitchell ED, Murray CC, Meads D, et al. (2017): Clinical and cost-effectiveness, safety and acceptability of community intravenous antibiotic service models: CIVAS systematic review. Bmj Open 7.
10. Nuutila J, Jalava-Karvinen P, Hohenthal U, et al. (2013): A rapid flow cytometric method for distinguishing between febrile bacterial and viral infections. J Microbiol Methods 92: 64-72.
11. Nuutila J, Jalava-Karvinen P, Hohenthal U, et al. (2013): Use of complement regulators, CD35, CD46, CD55, and CD59, on leukocytes as markers for diagnosis of viral and bacterial infections. Hum Immunol 74:522-530.
12. Nuutila J, Jalava-Karvinen P, Hohenthal U, et al. (2014): Bacterial infection (BI)-INDEX: an improved and simplified rapid flow cytometric bacterial infection marker. Diag Microbiol Infect Dis 78: 116-126.
13. Zhu GJ, Zhu J, Song L, et al. (2015): Combined use of biomarkers for distinguishing between bacterial and viral etiologies in pediatric lower respiratory tract infections. Infect Dis 47: 289-293.
14. ten Oever J, Netea MG, Kullberg BJ (2016): Utility of immune response-derived biomarkers in the differential diagnosis of inflammatory disorders. J Infect 72: 1-18.
15. Mokuda S, Doi O, Takasugi K (2012): Simultaneous quantitative analysis of the expression of CD64 and CD35 on neutrophils as markers to differentiate between bacterial and viral infections in patients with rheumatoid arthritis. Mod Rheumatol 22: 750-757.
16. Stalhammar ME, Sindelar R, Hakansson LD (2016): Neutrophil Receptor Response to Bacterial N-formyl Peptides is Similar in Term Newborn Infants and Adults in Contrast to IL-8. Scan J Immunol 84: 332-337.
17. Simon AK, Hollander GA, McMichael A (2015): Evolution of the immune system in humans from infancy to old age. Proc R Soc B-Biol Sci 282: 20143085.
18. Pansa P, Hsia Y, Bielicki J, et al. (2018): Evaluating Safety Reporting in Paediatric Antibiotic Trials, 2000-2016: A Systematic Review and Meta-Analysis. Drugs 78: 231-244.
19. Du J, Li L, Dou Y, et al. (2014): Diagnostic Utility of Neutrophil CD64 as a Marker for Early-Onset Sepsis in Preterm Neonates. PLoS One 9: e102647.
20. Ovrenovits M, Pakos EE, Vartholomatos G, et al. (2015): Flow cytometry as a diagnostic tool for identifying total hip arthroplasty loosening and differentiating between septic and aseptic cases. Eur J Orthop Surg Traumatol 25: 1153-1159.
21. Li S, Huang X, Chen Z, et al. (2013): Neutrophil CD64 expression as a biomarker in the early diagnosis of bacterial infection: a meta-analysis. Int J Infect Dis 17: E12-E23.
22. Nuutila J, Hohenthal U, Laitinen L, et al. (2007): Simultaneous quantitative analysis of Fc gamma RI (CD64) expression on neutrophils and monocytes: A new, improved way to detect infections. J Immunol Methods 328: 189-200.
23. Jamsa J, Ala-Kokko T, Huotari V, et al. (2018): Neutrophil CD64, C-reactive protein, and procalcitonin in the identification of sepsis in the ICU – Post-test probabilities. J Crit Care 43: 139-142.
24. Nuutila J, Hohenthal U, Laitinen I, et al. (2006): Quantitative analysis of complement receptors, CR1 (CD35) and CR3 (CD11b), on neutrophils improves distinction between bacterial and viral infections in febrile patients: Comparison with standard clinical laboratory data. J Immunol Methods 315: 191-201.