Research Article

Isil Cakir*, Nuri Cakir, Mustafa Altay Atalay and Ayse Nedret Koc

Large unstained cells are correlated with inflammatory biomarkers in patients with invasive aspergillosis

Büyük boyanmamış hücreler invaziv aspergillozis hastalarında enflamatuvar biyobelirteçler ile koreledir

Objective: The percentage of large unstained cells (%LUCs) reflects peroxidase-negative cells and activated lymphocytes. Unlike other infections, the value of %LUCs in the diagnosis of fungal infections is not clear. We aimed to evaluate %LUCs and its correlations with other inflammatory parameters of invasive aspergillosis (IA) patients.

Methods: Twenty patients and 20 healthy participants were included. Full blood count parameters including %LUCs values were recorded. Platelet to lymphocyte ratio (PLR), neutrophil to lymphocyte ratio (NLR), monocyte to lymphocyte ratio (MLR), eosinophil to lymphocyte ratio (ELR) were calculated.

Results: There was a significant difference between the study groups for %LUCs [2.40 (2.22–3.25); 1.43 (1.25–2.10), respectively; p < 0.001]. Furthermore, %LUCs were statistically significantly correlated with PLR, NLR and MLR (p = 0.020, 0.040, 0.040; respectively) but not correlated with ELR (p > 0.05).

Conclusion: The %LUCs values were significantly increased and correlated with markers of inflammation in patients. We suggest that the %LUCs is a useful predictor and may be an aid in the diagnosis and/or the management of IA and may help clinicians for follow up these patients in therapy process. Our study provides target pathways for further studies in the diagnosis of Aspergillus-infected patients using inflammatory parameters.

Keywords: Invasive aspergillosis; Large unstained cells; Inflammation; Platelet to lymphocyte ratio; Neutrophil to lymphocyte ratio; Monocyte to lymphocyte ratio; Eosinophil to lymphocyte ratio.
Introduction

Aspergillus species are found widespread in our environment. They can adapt to environmental conditions because they produce environmentally persistent sexual ascospores and asexual conidia. So they also become stress tolerant [1]. Aspergillus genus has several 100 species. But infections are mostly caused by Aspergillus fumigatus, Aspergillus flavus, Aspergillus nidulans, Aspergillus niger and Aspergillus terreus. More than 90% of infections are caused by A. fumigatus [2]. In immunocompetent people, neutrophils and macrophages of the innate immune system can eliminate the conidia of pathogenic Aspergillus strains which are inhaled. But, in immunocompromised people, Aspergillus may cause a variety of infectious diseases and allergic reactions. These infections can be followed by invasive aspergillosis (IA), known as the dissemination to other organs. There are also non-invasive forms named as allergic broncho pulmonary aspergillosis (ABPA) and aspergilloma [3]. Aspergillus may progress to lethal invasive infection due to the host’s immunological status, pulmonary structure and/or the fungal strains’ virulence. Although new therapeutic strategies are available, mortality rate is still very high. By Nucci et al. [4], the survival rate of Aspergillus infected patients has been told to increase with early diagnosis. But there are not enough easily accessible and cheap diagnostic biochemical tests for detecting this potentially deadly infection.

The percentage of large unstained cells (%LUCs) is a differential count parameter that reflects peroxidase-negative cells which do not fit into lymphocytes, neutrophils, monocytes, eosinophils and basophils [5]. %LUCs may also reflects activated lymphocytes. Platelet to lymphocyte ratio (PLR), neutrophil to lymphocyte ratio (NLR), monocyte to lymphocyte ratio (MLR) and eosinophil to lymphocyte ratio (ELR) are named as ‘systemic inflammation biomarkers’ in recent studies [6, 7]. Especially the PLR and NLR have been studied widely in various infectious diseases. However, their correlation with %LUCs and Aspergillus infection is unknown and unlike other infectious diseases, little is published about %LUCs, PLR, NLR, MLR and ELR in patients with IA.

Materials and methods

Study groups

The study comprised 20 patients (mean age 42±20) and 20 sex and age-matched controls (mean age 41.65±17.75). All of the patients have underlying diseases such as lung cancer, colon cancer, breast cancer, acute lymphoblastic leukaemia and renal transplantation (Table 1). Control group participants were selected from outpatient clinics and according to their laboratory test results infection and/or inflammation status was eliminated.

We retrospectively evaluated haematological parameters included %LUCs which were measured with Siemens ADVIA 2120 haematology analyzer, and calculated PLR, NLR, MLR and ELR levels of each participants in two groups.

Cases

Between July 2011 and May 2012, we retrospectively studied 20 patients treated for IA infections and 20 age and sex-matched healthy control participants who were admitted to Erciyes University Hospital of Kayseri, Turkey. We included patients who had contracted proven or probable IA defined by the revised European Organisation for Research and Treatment of Cancer-Mycosis study group (EORTC-MSG) criteria [8]. Various freshly taken clinical specimens of the 20 patients treated for proven or probable IA infections in Erciyes University Gevher Nesibe Hospitals clinics were analysed.

The identification of Aspergillus strains isolated from various clinical samples included both macroscopic and microscopic characteristics [9, 10]. The colonies growing in Sabouraud dextrose agar (SDA) were inoculated into potato dextrose agar to increase conidium and pigment productions and into Czapek-Dox Agar for the identification of species. For each of the growing species, the colony colour, morphology and growth characteristics at 25°C, 35°C and 44°C were recorded. For microscopic examination, lactophenol cotton blue was used for making a preparation, and the form and colour of conidia, the number of
sterigmata, shape of vesicles, structure of conidiophores, and presence and shape of “Hulle cells” were examined.

This preliminary identification was confirmed through more detailed studies using the molecular methodology of DNA sequencing analysis [11, 12]. As a reference method for species designation, sequencing of the internal transcribed spacer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) regions flanking 5.8S ribosomal DNA was performed for all of the isolates studied. The following thermal conditions were used: 94°C for 3 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min and 30 s, followed by a final extension step at 72°C for 10 min. Amplicons were purified with a MinElute PCR purification kit (QIAGEN, Germany) and sequenced on both strands with primer ITS1 or ITS4 and a BigDye Terminator cycle sequencing kit (Applied Biosystems, USA) on an ABI Prism 3100 genetic analyzer (Applied Biosystems, USA) [13]. The elongation PCR products were sequenced and species identification was performed by searching databases with the BLAST sequence analysis tool http://www.ncbi.nlm.nih.gov/BLAST/. The isolate was assigned to a species if it had 99% sequence homology with a sequence entry available in the searched databases (Table 2).

The study was approved by Erciyes University Research Ethics Committee (Resolution number: TSA-11-3244), Kayseri, Turkey.

Table 1: Demographic characteristics and laboratory findings of patients and control groups.

|                      | Patients          | Controls          | p-Value |
|----------------------|-------------------|-------------------|---------|
| Age (years)          | 42 ± 20b         | 41.65 ± 17.75a    | >0.05   |
| Gender (female/male) | 8/12              | 8/12              | -       |
| Underlying diseases  |                   |                   |         |
| Lung cancer          | 10                |                   |         |
| Colon cancer         | 2                 |                   |         |
| Breast cancer        | 1                 |                   |         |
| Acute lymphoblastic leukaemia | 4          |                  |         |
| Renal transplantation | 3                 |                   |         |
| Site of fungal infection |              |                   |         |
| Lung                 | 18                |                   |         |
| Eye                  | 2                 |                   |         |
| WBC                  | 11.99 (7.95–15.61)| 7.30 (6.66–9.31)  | 0.024a  |
| Neutrophil (%)       | 64.55 (55.85–81.25)| 57.50 (52.25–60.62)| 0.019a  |
| Eosinophil (%)       | 1.67 (0.20–2.57)  | 1.90 (1.50–3.05)  | 0.203   |
| Lymphocyte (%)       | 21.80 (10.60–32.02)| 32.05 (29.62–36.50)| 0.004a  |
| Monocyte (%)         | 5.95 (4.72–6.65)  | 7.90 (6.57–8.40)  | 0.001a  |
| Platelet             | 282 (204–410)     | 282 (215–323)     | 0.779   |
| Plateletcrit         | 0.26 (0.18–0.36)  | 0.30 (0.23–0.30)  | 0.076   |
| CRP                  | 123 (48–185)      | 8.05 (3.93–10.70) | <0.001a |
| %LUCs                | 2.40 (2.22–3.25)  | 1.43 (1.25–2.10)  | <0.001a |
| PLR                  | 16.73 (8.95–29.43)| 8.07 (6.03–10.08) | 0.002a  |
| NLR                  | 2.99 (1.74–7.82)  | 1.83 (1.46–2.08)  | 0.007a  |
| MLR                  | 0.28 (0.19–0.74)  | 0.25 (0.18–0.27)  | 0.106   |
| ELR                  | 0.09 (0.03–0.15)  | 0.06 (0.04–0.09)  | 0.303   |

%LUCs, Large unstained cells; PLR, platelet to lymphocyte ratio; NLR, neutrophil to lymphocyte ratio; MLR, monocyte to lymphocyte ratio; ELR, eosinophil to lymphocyte ratio. Mann-Whitney U-test was used; data are median and interquartile range (25%–75%). *Statistically significant, p < 0.05. bMean ± SD.

Table 2: DNA sequencing analysis of patients’ specimens.

| Specimen                        | Aspergillus fumigatus | Aspergillus niger | Aspergillus flavus | Aspergillus terreus |
|---------------------------------|-----------------------|-------------------|--------------------|--------------------|
| Bronchoalveolar lavage (BAL)    | 3                     | 2                 | 4                  | –                  |
| Pulmonary tissue                | 4                     | 1                 | 3                  | 1                  |
| Eye tissue                      | 2                     | –                 | –                  | –                  |
| Total number of patients        | 9                     | 3                 | 7                  | 1                  |
Statistical analysis

Statistical analysis was carried out using SPSS software version 23.0 for Windows (SPSS Inc., USA). Mann-Whitney U-test were used to determine significant differences between the groups. The results of groups were presented as median and interquartile range (25%–75%). To determine the relationship between the variables of each group, Spearman's correlation coefficients were performed. A receiver operating characteristic (ROC) analysis was used to select the best cut-off values. The p values ≤0.05 of the obtained results were accepted as statistically significant.

Results

Patients' %LUCs, PLR, NLR, MLR and ELR levels were numerically higher than controls'. In addition, statistically significant differences were found between the Aspergillus-infected and -uninfected groups for %LUCs, PLR and NLR. But there were no statistically significant differences for MLR and ELR levels (Table 1).

There were statistically significant correlations between patients' %LUCs and their PLR, NLR and MLR levels (p<0.05), but we did not find a correlation between %LUCs and ELR levels (Table 3). According to the ROC analysis of studied parameters, optimal cut-off points were decided using the maximum value of Youden’s index (sensitivity + specificity – 1). Cut-off values were identified: for %LUCs of 1.825 (AUC: 0.974; sensitivity 95%; specificity 85%); for PLR of 11.315 (AUC: 0.79; sensitivity 75%; specificity 95%), for NLR of 2.380 (AUC: 0.75; sensitivity 65%; specificity 85%), for MLR of 0.320 (AUC: 0.649; sensitivity 45%; specificity 95%), for ELR of 0.105 (AUC, 0.595; sensitivity 45%; specificity 80%) (Figure 1).

Discussion

Patients who are immunocompromised have a higher risk of invasive fungal infections (IFI) [14]. These infections in patients with neutropenia are life-threatening. Early detection and prompt initiation of antifungal therapy are critical factors that could decrease mortality [15]. Culture or histopathological evaluation of clinical specimens are the gold standard methods for the diagnosis of IFI, but the methods are time-consuming in cases of IFI [16].

Non-invasive diagnostic methods such as the detection of 1,3-β-D-glucan (BDG), and galactomannan (GM) or molecular biology techniques can be helpful for IFI diagnosis in the serum or plasma of high risk patients and can be helpful for IFI diagnosis, but the methods are time-consuming too and can not always be performed in cases of IFI, as patients may be unstable, hypoxic or with coagulopathy [17].

Indeed, the use of biomarkers such as procalcitonin (PCT), C-reactive protein (CRP) and fibrinogen could lead to a suspicion of IFI in high-risk haematology patients and in particular, in patients with prolonged neutropenia [18].

In recent studies the prognostic value of the %LUCs has been shown in patients with other infectious diseases. Vanker and Ipp [5] showed a significant increase in the %LUCs levels in asymptomatic, untreated, HIV-infected group and they reported a correlation between %LUCs and markers of immune activation and CD4 counts and they conclude that, the %LUCs may be of value in identifying

| %LUCs | Correlation coefficient | p-Value |
|-------|-------------------------|---------|
| PLR   | -0.496                  | 0.020*  |
| NLR   | -0.450                  | 0.040*  |
| MLR   | -0.454                  | 0.040*  |
| ELR   | -0.230                  | 0.328   |

*Statistically significant, p < 0.05.
HIV-infected patients at risk of disease progression. A recent study by Vanker and Ipp [19] showed again the significant differences of %LUCs in HIV-infected individuals and mentioned the correlation with markers of immune activation. In another study reported by Shin et al. [20] showed that in varicella patients %LUC was significantly increased and corresponded with clinical improvements.

Compared to other inflammatory markers, the PLR, NLR, MLR and ELR are simple, widely available and inexpensive tests. They are indicators of immune response-related diseases. Recent studies have confirmed that they are related to the diagnosis, progression and prognosis of various clinical conditions including thrombosis-related diseases, cardiovascular diseases, infections, cancers and polips, etc. [21–26]. In our retrospective study, for the first time, we determined the levels of inflammatory markers and %LUCs, also we evaluated whether there were independent relationships between markers of inflammation (PLR, NLR, MLR and ELR) and %LUCs in patients with Aspergillus infection.

Our study found statistically significantly higher %LUCs, PLR and NLR levels and numerically higher MLR and ELR levels in the Aspergillus-infected group (Table 1). Although the relationship between parameters were not very strong, the correlations between %LUCs and PLR, NLR and MLR were significant, suggesting that these inflammatory parameters may serve as useful markers in diagnosis of Aspergillus-infected patients and may help clinicians for follow up these patients in therapy process (Table 3). The results from ROC analysis of our data suggested that the use of cut-off values for %LUCs, PLR, NLR, MLR and ELR (1.825, 11.315, 2.380, 0.320 and 0.105, respectively) would be optimum for clinical use. So, values above these numbers may be independent confirmative predictors of patients with Aspergillus infection (Figure 1).

Conclusion

Due to significant elevations of %LUCs, PLR and NLR in patients group, these markers can be used as confirmatory markers for Aspergillus-infected patients. Therefore, especially the %LUCs may be of value in diagnosis of Aspergillus infection. Our study limitation included the relatively small number of participants. New studies are required to evaluate the value of %LUCs in predicting Aspergillus-infected patients and to confirm our findings.

Acknowledgements: The authors wish to thank all participating staff in Microbiology Department of Erciyes University.

Conflict of interest statement: No conflict of interest was declared by the authors.

References

1. Wyatt TT, Golovina EA, van Leeuwen R, Hallsworth JE, Wösten HA, Dijksterhuis J. A decrease in bulk water and mannitol and accumulation of trehalose and trehalose-based oligosaccharides define a two-stage maturation process towards extreme stress resistance in ascospores of Neosartorya fischeri (Aspergillus fischeri). Environ Microbiol 2015;17:383–94.
2. Perfect JR, Cox GM, Lee JY, Kaufman CA, de Repentigny L, Chapman SW, et al. The impact of culture isolation of Aspergillus species: a hospital-based survey of aspergillosis. Clin Infect Dis 2001;33:1824–33.
3. Kosmidis C, Denning DW. The clinical spectrum of pulmonary aspergillosis. Thorax 2015;70:270–7.
4. Nucci M, Nouër SA, Cappone D, Anaissie E. Early diagnosis of invasive pulmonary aspergillosis in hematologic patients: an opportunity to improve the outcome. Haematologica 2013;98:1657–60.
5. Vanker N, Ipp H. The use of the full blood count and differential parameters to assess immune activation levels in asymptomatic, untreated HIV infection. S Afr Med J 2013;104:45–8.
6. Park BK, Park JW, Han EC, Ryoo SB, Han SW, Kim TY, et al. Systemic inflammatory markers as prognostic factors in stage IIA colorectal cancer, J Surg Oncol 2016;114:216–21.
7. Wu Y, Chen Y, Yang X, Chen L, Yang Y. Neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) were associated with disease activity in patients with systemic lupus erythematosus. Int Immunopharmacol 2016;36:94–9.
8. Verweij PE, Brandt ME. Aspergillus, fusarium, and other opportunistic monilaceous fungi. In: Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA, editors. Manual of clinical microbiology, 9th ed., vol. 2. Washington: ASM Press, 2007:1802–38.
9. Ascioglu S, Rex JH, de Pauw B, Bennett JE, Bille J, Crokaert F, et al. Defining opportunistic invasive fungal infections in immunocompromised patients with cancer and hematopoietic stem cell transplants: an international consensus. Clin Infect Dis 2002;34:7–14.
10. De Pauw B, Walsh TJ, Donnelly JP, Stevens DA, Edwards JE, Calandra T, et al. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. Clin Infect Dis 2008;46:1813–21.
11. Mandiviwala T, Shinde R, Kalra A, Sobel JD, Akins RA. High-throughput identification and quantification of Candida species using high resolution derivative melt analysis of panfungal amplicons. J Mol Diagn 2010;12:91–101.
12. Balajee SA, Sigler L, Brandt ME. DNA and the classical way: identification of medically important molds in the 21st century. Med Mycol 2007;45:475–90.
13. Sanguinetti M, Porta R, Sali M, La Sorda M, Pecorini G, Fadda G, et al. Evaluation of VITEK 2 and RapID yeast plus systems for yeast species identification: experience at a large clinical microbiology laboratory. J Clin Microbiol 2007;45:1343–6.
14. Pagano L, Akova M, Dimopoulos G, Herbrecht R, Drgona L, Blijlevens N. Risk assessment and prognostic factors for mould-related diseases in immunocompromised patients. J Antimicrob Chemother 2011;66(Suppl 1):i5–14.
15. Pagano L, Caira M, Candoni A, Offidani M, Fianchi L, Martino B, et al. The epidemiology of fungal infections in patients with hematologic malignancies: the SEIFEM-2004 study. Haematologica 2006;91:1068–75.
16. Del Bono V, Mikulska M, Viscoli C. Invasive aspergillosis: diagnosis, prophylaxis and treatment. Curr Opin Hematol 2008;16:586–93.
17. Hörmet Öz HT, Koç AN, Atalay MA, Eser B, Yıldız O, Kaynar L. The diagnostic value of the galactomannan and (1,3)_beta-d-glucan in diagnosis of invasive Aspergillosis. Nobel Medicus 2014;10:44–9.
18. Roques M, Chretien ML, Favennec C, Lafon I, Ferrant E, Legouge C, et al. Evolution of procalcitonin, C-reactive protein and fibrinogen levels in neutropenic leukaemia patients with invasive pulmonary aspergillosis or mucormycosis. Mycoses 2016;59:383–90.
19. Vanker N, Ipp H. Large unstained cells: a potentially valuable parameter in the assessment of immune activation levels in HIV infection. Acta Haematol 2014;131:208–12.
20. Shin D, Lee MS, Kim DY, Lee MG, Kim DS. Increased large unstained cells value in varicella patients: a valuable parameter to aid rapid diagnosis of varicella infection. Dermatol 2015;42:795–9.
21. Yang W, Liu Y. Platelet-lymphocyte ratio is a predictor of venous thromboembolism in cancer patients. Thromb Res 2015;136:212–5.
22. Yayla C, Akboga MK, Canpolat U, Akyel U, Gayrettli K, Doğan M, et al. Platelet to lymphocyte ratio can be a predictor of infarct-related artery patency in patients with ST-segment elevation myocardial infarction. Angiology 2015;66:831–6.
23. Meng X, Wei G, Chang Q, Peng R, Shi G, Zheng P, et al. The platelet-to-lymphocyte ratio, superior to the neutrophil-to-lymphocyte ratio, correlates with hepatitis C virus infection. Int J Infect Dis 2016;45:72–7.
24. Aydin E, Karadag MA, Cecen K, Cigsar G, Aydin S, Demir A, et al. Association of mean platelet volume and the monocyte/lymphocyte ratio with brucella-caused epididymo-orchitis. Southeast Asian J Trop Med Public Health 2016;47:450–6.
25. Sun X, Liu X, Liu J, Chen S, Xu D, Li W, et al. Preoperative neutrophil-to-lymphocyte ratio plus platelet-to-lymphocyte ratio in predicting survival for patients with stage I-II gastric cancer. Chin J Cancer 2016;35:57.
26. Brescia G, Pedruzzi B, Barion U, Marioni G. Are neutrophil-, eosinophil-, and basophil-to-lymphocyte ratios useful markers for pinpointing patients at higher risk of recurrent sinonasal polyps? Am J Otolaryngol 2016;37:339–45.