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Number of Counting Cells and Cytospins Selection Influences on Bronchoalveolar Lavage Cell Profiles

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A B S T R A C T

Bronchoalveolar lavage (BAL) fluid cells count provides information about presence or absence of interstitial lung diseases. BAL fluid samples were taken from 50 patients hospitalized in University Hospital for Lung Diseases »Jordanovac« in Zagreb, Croatia. The samples of BAL fluid were prepared by cytocentrifuge. From each sample two cytospin were selected (C1 and C2) and after determing adequacy, counted up to 200 and 400 cells. After air drying, samples were stained according to May Grünwald Giemsa (MGG). Cells were counted by light microscope at magnification of 400x. Obtained results were analyzed in Statistics version 6 and Med Calc. Results for bronchial epithelial cells, alveolar macrophages, lymphocytes and neutrophilic granulocytes showed insignificant statistical differences between groups ($p>0.05$). Eosinophils percentages showed borderline insignificant statistical difference between groups of these cells ($p=0.052$.). As it was exemplificated, the percentages of differentiated cells do not significant differ according to differentiation on 200 and 400 cells and cytospin selection.

Key words: *bronchoalveolar lavage, differentiation, cytocentrifuge, cytospin, statistic, quality*

Introduction

Bronchoalveolar lavage (BAL) is diagnostic bronchoscopic method based on washing of lower parts of respiratory tract and the alveolar spaces with 0.9% isotonic solution[1]. BAL fluid analysis and cell counting is every day routine in Cytological Laboratory. Quantitative changes of cells which may be noticed by differentiation, provides unique information about presence or absence of interstitial lung diseases[2–6]. Well-preserved cells are counting in light microscope under magnification 400x, minimally up to 200 and if it is required, by the number of 500. Some authors are mentioning counting 1,000 cells in cytospin[1,7]. Cells that are counting are alveolar macrofages, lymphocytes, neutrophils, eosinophils, mast cells and plasma cells, if present. Assigned numbers have to be expressed as percentages. Among the first 100 cells, bronchial epithelial cells have to be count. Their number is also expressing as percentage, and it is sign for sample adequacy. Enlarged number of inflammatory cells in BAL fluid is calling alveolitis. Regarding predominant cells in BAL fluid, it is possible to distinguish these types of alveolitis: lymphocytic, neutrophilic, eosinophilic and mixed type of alveolitis[8]. The aim of this study is to conclude if there are some significant differences in quantitative relations between percentages of cells interpreted by BAL fluid differentiation on 200 and 400 cells and through two slides, cytospin 1 (C1) and cytospin 2 (C2), made from the same sample and prepared in the same way.

Materials and Methods

The samples taken in siliconised test tubes were filtered by filter paper, pores 34 μm, to eliminate excess of mucus. After that, we used 100 μl filtered material for each cytocentrifuge chamber (Termo Shandon Cytospin 4) and centrifuged them in a speed of 1,000 rpm in 5 min. From each sample of BAL fluid cytospin 1 and cytospin 2 were prepared. Cytospins were air-dried for two hours and stained according to May Grünwald Giemsa (MGG)

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procedure in Sakura Tissue TekDrs autostainer. MGG staining procedure is as follows: May Grünwald for 3 minutes, Giemsa (dilution 1:20) 17 minutes, deionized water (pH 6.8) for 2 minutes, deionized water (pH 6.8) for 4 minutes[9].

Differentiation of BAL fluids was done by five cytotehnologists. The cells were differentiated by light microscope under magnification 400 times[10]. In all cytospins were differentiated 200 and 400 cells[1,7–11]. In cytospin 1 and cytospin 2 that belong to the same BAL fluid, 200 and 400 cells were counting by the same cytotehnologist. Differentiation was done through several fields until 200 cells, and the same procedure was repeated by the number of 400 cells in cytospin 1 and cytospin 2 for every sample of BAL fluid. Differentiation was directed to the center of cytospin[12]. The cells were counting in circular pattern from left to right until wanted number of cells was obtain, watching that the edge of previous microscopic field overlaps with the beginning edge of the next field. The cells at peripheral edges of microscopic fields were not counting. In that way, neither one cell was missed. The percentages of cells were calculated by formula: number of cells x100/200 (or 400).

Statistical analysis

The numerical values were analyzed in Statistics version 6 and Med Calc. Descriptive statistics were used for attributes and measured variables description and for their version in tables. If variables were following normal distribution were used mean ($\overline{X}$) an standard deviation (SD), or median and interquartile range (IQR) in opposite. Distribution normality was tested by Kolmogor-Smirn test. Cronbach alpha and inter-item correlation coefficient were used for testing coincidence, certainty and internal compactness of multiple measurements for the same variable whit normal distribution, while for testing differences among multiple measurements was used Anova. For testing coincidence and certainty of multiple measurements for the same variable that is not following normal distribution, Kendall coefficient of concordance has been performed, while Friedman Anova (Anova F) was used for testing differences among multiples measurements. As statistical significant difference $p<0.05$ has been calculated.

Results

Results obtained by differentiation BAL fluid samples of 50 patients, 28 man and 22 women were included in the study. Age range of patients is 22–27 years, $\overline{X}$±SD of years is 50.56±13.30. From each sample, 200 and 400 cells of two cytospins that were marked as cytospin 1 and cytospin 2 have been differentiated. Percentage range of macrophages is 15.75% to 97.50% from total cell count, percentage range of lymphocytes is 1–78.75%, percentage range of neutrophils is 0.00%–79%, eosinophils 0.00%–57%, bronchial epithelial cells from 0.00%–9.00%. In four samples, percentages of bronchial epithelial cells indicated adequate BAL fluid in cytospins 1, while in cytospins 2 of the same BAL fluid, percentages of bronchial epithelial cells were more than 7% and indicated inadequate BAL fluid sample. Anyhow, mentioned four samples of BAL fluid were used in our research because mean percentage of bronchial epithelial cells of those samples was still ≤7%. Results showed that variables ($\overline{X}$ and SD) for bronchial epithelial cells, macrophages and lymphocytes are following normal distribution. Differences in $\overline{X}$±SD of bronchial epithelial cells between cytospins were statistical insignificant. Their results are for C1 3.66±2.22% and C2 3.68±2.24%. Median of bronchial epithelial cells for C1 and C2 is 4.0%.

Coincidence coefficient (0.7562) and significant level ($p=0.928056$) indicate insignificant differences between bronchial epithelial cells (Table 1).

$\overline{X}$±SD of macrophages counted on 200 cells in C1 is 60.78±20.90%, in C2 is 60.86±21.30%, and on 400 cells in C1 is 60.77±21.20%, in C2 is 60.92±21.28%. Median of macrophages counted on 200 cells in C1 is 60.25%, in C2 is 59.75%. Median of macrophages counted on 400 cells in C1 and C2 is 59.38%. Confidence interval of 95% when counting on 200 cells, in C1 is 54.84–66.72, in C2 is 54.81–66.91, while on 400 cells confidence interval is 54.74–66.80 in C1, in C2 is 54.87–66.96. Inter-item correlation test (0.974572), significant level ($p=0.997287$) and Anova F (0.015777) showed statistical insignificant differences between alveolar macrophages percentages among investigated groups (Table 2).

According to percentage of lymphocytes, results showed that from 50 samples of BAL fluid, 29 (58%) had more than 15% of lymphocytes in C1 and C2 differentiated on 200 and 400 cells while 19 (38%) samples had less than 15% of lymphocytes, also differentiated on 200 and 400 cells in C1 and C2. Two samples of BAL fluid had more than 15 % of lymphocytes in C1 while in C2 prepared of the same BAL fluid sample had less than 15% of lymphocytes and were not categorized.

TABLE 1
BRONCHIAL EPITHELIAL CELLS PERCENTAGES IN IVESTIGATED GROUPS AND STATISTICAL SIGNIFICANCE OF DIFFERENCES (COEFF. OF CONCORDANCE=0.7562, P=0.928056)

	Cytospin	Valid No	$\overline{X}$	Confidence –95%	Confidence +95%	Median	Min	Max	SD
BE 100	C1	50	3.66	3.03	4.29	4.00	0.00	8.00	2.22
	C2	50	3.68	3.04	4.32	4.00	0.00	9.00	2.24

BE – bronchial epithelial cells, C1 – cytospin 1, C2 – cytospin 2, $\overline{X}$ – mean, Min – minimum, Max – maximum, SD – standard deviation

TABLE 2
ALVEOLAR MACROPHAGES PERCENTAGES IN INVESTIGATED GROUPS AND STATISTICAL SIGNIFICANCE OF DIFFERENCES (AVERAGE INTER-ITEM CORR=0.974572, ANOVA F=0.015777, P=0.997287)

	Cytospin	Valid No	$\overline{X}$	Confidence –95%	Confidence +95%	Median	Min	Max	SD
MF 200	C1	50	60.78	54.84	66.72	60.25	19.00	97.50	20.90
	C2	50	60.86	54.81	66.91	59.75	18.50	96.00	21.30
MF 400	C1	50	60.77	54.74	66.80	59.38	15.75	97.25	21.20
	C2	50	60.92	54.87	66.96	59.38	18.50	95.50	21.28

MF – alveolar macrophages, C1 – cytospin 1, C2 – cytospin 2, $\overline{X}$ – mean, Min – minimum, Max – maximum, SD – standard deviation

$\overline{X}$±SD of lymphocytes were not significantly different between cytospins counted on 200 cells (C1=26.37±21.17%, C2=26.95±21.59%) from cytospins of the same sample of BAL fluid, counted on 400 cells (C1=26.39±21.44%, C2=26.52±21.50%). Median for cytospins counted on 200 cells in C1 is 20.25%, in C2 is 23.00%, for cytospins counted on 400 cells, in C1 is 20.63%, and in C2 is 21.00%. Confidence intervals of 95% of lymphocytes counted on 200 cells are: 20.35–32.39 in C1, 20.81–33.09 in C2, counted on 400 cells are 20.29–32.48 in C1, 20.41–32.62 in C2. Average inter-item correlation test (0.980280), p=0.836708, and Anova F (0.284255) showed statistical insignificant differences between lymphocytes percentages (Table 3).

Variables of neutrophilic and eosinophilic granulocytes (median and IQR) are not following normal distribution. Obtained results for neutrophils showed $\overline{X}$±SD counted on 200 cells (9.78±13.48% in C1, 9.47±13.59% in C2), and $\overline{X}$±SD counted on 400 cells (9.91±13.75% in C1, 9.69±13.99% in C2). Median of neutrophils counted on 200 cells in C2 and median of these cells counted by the number of 400 in C1 are equal (4.50%). Median for neutrophils counted on 200 cells in C1 is 4.00%, for neutrophils counted on 400 cells in C2 is 4.75%. Confidence intervals of 95% in C1, C2 counted on 200 cells are 5.95–13.61, 5.61–13.33, and in C1, C2 counted on 400 cells are 6.00–13.81, 5.71–13.67. Coincidence coefficient of neutrophils (0.99518) and p=0.86772 showed statistical insignificant differences in Anova test (Table 4). During differentiation, in C1 of 15 BAL fluid samples and in C2 of 14 BAL fluid samples, eosinophils were not present. Differences in $\overline{X}$±SD percentages of eosinophils between cytospins are little higher than between previous groups of cells (C1, C2 (200)=3.07±8.81%, 2.81±8.75%; C1, C2 (400)=2.96±8.85%, 2.88±8.72%). Medians in C1 and C2 counted on 200 and in C2 counted on 400 are equal (0.75%), while in C1 counted on 400 cells is 0.50%. Confidence interval of 95% for eosinophils counted on 200 cells is 0.57–5.57 in C1, 0.32–5.30 in C2, and counted on 400 cells is 0.44–5.48 in C1, 0.40–5.36 in C2. Coincidence coefficient (0.94876), significant level (p=0.05288), showed borderline statistical insignificant difference (Table 5).

TABLE 3
LYMPHOCYTES PERCENTAGES IN IVESTIGATED GROUPS AND STATISTICAL SIGNIFICANCE OF DIFFERENCES (AVERAGE INTER-ITEM CORR=0.980280, ANOVA F=0.284255 P=0.836708)

	Cytospin	Valid No	$\overline{X}$	Confidence –95%	Confidence +95%	Median	Min	Max	SD
Ly 200	C1	50	26.37	20.35	32.39	20.25	1.00	74.50	21.17
	C2	50	26.95	20.81	33.09	23.00	1.00	77.00	21.59
Ly 400	C1	50	26.39	20.29	32.48	20.63	1.25	78.75	21.44
	C2	50	26.52	20.41	32.62	21.00	1.00	76.25	21.50

Ly – lymphocytes, C1 – cytospin 1, C2 – cytospin 2, $\overline{X}$ – mean, Min – minimum, Max – maximum, SD – standard deviation

TABLE 4
NEUTROPHILIC GRANULOCYTES PERCENTAGES IN INVESTIGATED GROUPS AND STATISTICAL SIGNIFICANCE OF DIFFERENCES (ANOVA χ^2=0.7232704, COEFF. OF CONCORDANCE=0.99518, P=0.86772)

	Cytospin	Valid No	$\overline{X}$	Confidence –95%	Confidence +95%	Median	Min	Max	SD
Ne 200	C1	50	9.78	5.95	13.61	4.00	0.50	73.00	13.48
	C2	50	9.47	5.61	13.33	4.50	0.00	75.50	13.59
Ne 400	C1	50	9.91	6.00	13.81	4.50	0.25	76.50	13.75
	C2	50	9.69	5.71	13.67	4.75	0.75	79.00	13.99

Ne – neutrophilic granulocytes, C1 – cytospin 1, C2 – cytospin 2, $\overline{X}$ – mean, Min – minimum, Max – maximum, SD – standard deviation

TABLE 5
EOSINOPHILIC GRANULOCYTES PERCENTAGES IN INVESTIGATED GROUPS AND STATISTICAL SIGNIFICANCE OF DIFFRENCES (ANOVA χ^2=7.685294, COEFF. OF CONCORDANCE=0.94876, P=0.05299)

	Cytospin	Valid No	$\overline{X}$	Confidence –95%	Confidence +95%	Median	Min	Max	SD
Eo 200	C1	50	3.07	0.57	5.57	0.75	0.00	57.00	8.81
	C2	50	2.81	0.32	5.30	0.75	0.00	57.00	8.75
Eo 400	C1	50	2.96	0.44	5.48	0.50	0.00	56.75	8.85
	C2	50	2.88	0.40	5.36	0.75	0.00	56.50	8.72

Eo – eosinophilic granulocytes, C1 – cytospin 1, C2 – cytospin 2, $\overline{X}$ – mean, Min – minimum, Max – maximum, SD – standard deviation

Discussion and Conclusion

Samples with less than 5% of bronchial epithelial cells were considered adequate BAL fluid samples for further differentiation[13]. Regarding every day experience and obtained results in our laboratory, we are considering presence up to 7% of bronchial epithelial cells in BAL fluid as an adequate sample for differentiation. Beside the bronchial epithelial cell percentage, it is important to evaluate presence of bronchial epithelial cell clusters and squamous epithelial cells as additional adequacy criterion. An extended microscopic screening of the BAL fluid cytocentrifuged preparations is recommended, in order to evaluate the presence of epithelial cells under low magnification (100x). For reliable differential count of bronchial epithelial cells differentiation on 500 cells is recommended, while for neutrophils, alveolar macrophages, lymphocytes and eosinophils differentiation on 300 cells is necessary but sufficient[14].

Regular careful manipulation with BAL fluid samples until they were transported to the laboratory and standardized technical preparation in laboratory are capital for differentiation of BAL fluid cells. Time from bronchioalveolar lavage procedure, taking samples and their receipt in laboratory should be minimized, in purpose of better cell preservation. In order to prevent cells adhesion on glass inside test tube in which is BAL fluid transported, it is important to impregnate test tubes with silikospray. Cytocentrifugation speed and duration of cytocentrifugation effect cells differentiation, specially lymphocytes and macrophages. The highest lymphocyte recovery was found at 1,200 rpm, during 10 minutes[15]. Well air-dried cytospin preparations are basic criterion for staining. In insufficient dried cytospins, clusters of cells, changed cell shapes, stain remains and detritus are present after staining, which may cause difficulties in differentiation of cells. Central area of well-prepared cytospin contains regular, monolayer distributed cells, which makes differentiation easier[12,14] and repress possibilities of cell replacement such as macrophages in macrophage-hystiocyte transformation with transformed lymphocyte cells, multinuclear macrophages with multinuclear giant cells. Clustered cells without well-seen borders should not be counted. That type of cytospin preparations and preparations with squamous epithelial cells are interpreting as inadequate BAL fluid samples. In purpose of samples quality evaluation, two cytospins from the same BAL fluid sample were differentiated. Cytospins that belong to the same BAL fluid were prepared by cytocentrifuge at the same time. Quantity of 100 μl was dropped in cytospin 1 first and than in cytospin 2. After centrifugation, cell distribution and their preservation in both cytospins were equal. There were insignificant differences between cytospins (C1 and C2) counted on 200 and 400 cells for macrophages, lymphocytes and neutrophils comparing C1 200 and C1 400 i.e. C2 200 and C2 400 according to the cell types in our study. All statistical combinations showed statistical insignificant differences of means ($p>0.05$). Eosinophils percentages showed borderline statistical differences between differentiated groups ($p=0.052$), which was expected because eosinophils are usually present in a small number in BAL fluid. According to that, small difference in a count of these cells may be statistically noticed. As it was exemplificated, the percentages of differentiated cells do not significant differ according to differentiation on 200 and 400 cells and cytospin selection.

REFERENCES

1. GEE JB, FISCH RB, Thorax, 35 (1980) 1. — 2. WELKER L, JÖRRES RA, COSTABEL U, MAGNUSSEN H, Eur Respir J, 24 (2004) 1000. — 3. ROTTOLI P, BARGAGLI E, Curr Opin Pulm Med, 9 (2003) 418. — 4. COSTABEL U, GUZMAN J, Curr Opin Pulm Med, 7 (2001) 255. — 5. KLECH H, HUTTER C, Eur Resp J, 3 (1990) 937. — 6. TEKAVEC TRKANJEC J, PEROŠ GOLUBIČIĆ T, SMOJVER JEŽEK S, ALILOVIĆ M, Lijec Vjesn, 125 (2003) 145. — 7. DE BRAUWER EIGB, JACOBS JA, NIEMAN FH, BRUGGEMAN CA, DRENT M, J Clin Microbiol, 37 (1999) 427. — 8. SMOJVER JEŽEK S, Citološke pretrage upalnih bolesti plućnog parenhima, In: PEROŠ GOLUBIČIĆ T, PAVLOVIĆ M (Ed) Tuberkuloza, pneumonija, pneumonitis-upalne bolesti plućnog parenhima (Medicinska naklada, Zagreb, 2002). — 9. HAUPTMAN E, ČREPINKO I, Osnove kliničke hematologije (Školska knjiga, Zagreb, 1991). — 10. KLECH H, POHL W, Eur Respir J, 2 (1989) 561. — 11. GOLDSTEIN RA, ROHATGI PK, BERGOFSKY EH, BLOCK ER, DANIELE RP, DANTZKER DR, Am Rev Respir Dis, 142 (1990) 481. — 12. DE BRAUWER E, Anal Quant Cytol Histol, 22 (2000) 143. — 13. WIELD LG, KEEBLER MC, KOSS GL, PATTEN FS, ROSENTHAL LD (Eds), Compendium on Diagnostic Cytology (Tutorials of Cytology, Chicago, Illinois, 1992). — 14. DE BRAUWER EI, JACOBS JA, NIEMAN F, BRUGGEMAN CA, DRENT M, Anal Quant Cytol Histol, 6 (2002) 337. — 15. DE BRAUWER EI, JACOBS JA, NIEMAN F, BRUGGEMAN CA, WAGENAAR SS, DRENT M, Anal Quant Cytol Histol, 5 (2000) 416.

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UTJECAJ UKUPNOG BROJA DIFERENCIRAJUĆIH STANICA I ODABIRA PREPARATA NA STANIČNI SASTAV BRONHOALVEOLARNOG LAVATA

S A Ž E T A K

Citološka analiza bronhoalveolarnog lavata (BAL) sastavni je dio rutinske obrade u citološkom laboratoriju. Kvantitativne promjene staničnog sastava BAL-a omogućuju informacije o prisustvu ili odsustvu plućnih bolesti. Analizirani su BAL-ovi 50 bolesnika hospitaliziranih u Klinici za plućne bolesti »Jordanovac«. Od svakog uzorka diferencirana su po dva citospina (C1 i C2) nakon procjene adekvatnosti i to na 200 i 400 stanica. Citospinovi su bojani May Grünwald Giemsa metodom i diferencirani uz pomoć svjetlosnog mikroskopa uz povećanje 400x. Dobiveni postoci stanica analizirani su statističkim programskim paketom Statistika, verzija 6.0, te programom Med Calc. Postoci stanica bronhalnog epitela, makrofaga, limfocita i neutrofilnih granulocita nisu pokazali statistički značajne razlike među ispitivanim skupinama ($p>0,05$). Granične razlike koje nisu statistički značajne primjećene su među skupinama u postocima eozinofilnih granulocita ($p=0,052$). Postoci diferenciranih stanica nisu se statistički značajno razlikovali obzirom na broj stanica koje su diferencirane (200 i 400), kao ni u različitim citospinovima istog BAL-a. Može se zaključiti da odabir preparata citospina i ukupni broj diferenciranih stanica ne mijenjaju nalaz staničnog sastava bronhoalveolarnog lavata.