Chapter 2

Use of Polylysine-coated slides in clinical bronchoalveolar lavage fluid samples

CFM Linssen, JA Jacobs, FHM Nieman, EIM Cornelissen, M. Drent

Analytical and Quantitative Cytology and Histology 2003;25:281-284

Abstract

Objective
Polylysine coating of microscope slides provides superior cell adhesion. We compared polylysine-coated (PLC) slides to conventional slides in cyto-centrifuged bronchoalveolar lavage (BAL) fluid samples.

Study design
Twenty BAL fluid (BALF) samples with representative numbers of alveolar macrohages (Ams); lymphocytes (Lyms) and polymorphonuclear neutrophils (PMNs) were cytocentrifuged on uncoated slides and on PLC slides (2 slides each). Cell density, differential cell counts and cytomorphology were assessed on May-Grünwald-Giemsa-stained preparations. Reliability of cell differentiation was expressed as a Φ value, which measures combined reproducibility and agreement. Statistical significance of differences between slides was calculated with ANOVA. Clinical relevance was assessed using a validated computer program predicting the most probable diagnosis.

Results
Although not statistically significant, cell recovery was lower on PLC slides as compared to uncoated slides. PLC slides held significantly fewer Lyms as compared to uncoated slides. (mean value $\pm$ SD: 25.89% $\pm$ 28.26 versus 28.34 % $\pm$ 29.96, respectively). Counts of AMs, 4ILyms and PMNs displayed excellent Φ values for both uncoated and PLC slides. No discrepancies in the computer-generated diagnoses were found.

Conclusion
For BALF cytology on cytocentrifuged preparations, PLC slides are not superior to conventional slides.

Introduction

The differential cell count obtained by bronchoalveolar lavage (BAL) gives valuable information in the assessment of interstitial lung diseases and pneumonia[1]. Easy-to-read monolayer preparations that reveal well-preserved cell morphology can be obtained by cytocentrifugation. The cytocentrifugation process involves low-speed centrifugation of BAL fluid (BALF), during which the cells are deposited on the microscope slide and the cell-free fluid is absorbed into a filter path.

BALF differential cell counts on cytocentrifuged preparations, however, underestimate the proportion of lymphocytes (Lyms)[2]. In a previous study, we demonstrated that this difference was influenced by the operating conditions of the cytocentrifuge[3]. In the present study, we evaluated the recovery of different BALF cell types when cytocentrifuged on polylysine-coated (PLC) slides. PLC slides have a positively charged surface that provides superior cell adhesion. They have been used in the cytology of buccal smears and of urine and cerebrospinal fluid samples[4-6] but have never been evaluated for cytocentrifugation of BALF samples. We compared PLC slides to conventional glass slides with respect to cell recovery, differential cell count and cytomorphology and assessed the clinical relevance of the differences.

Materials and methods

BALF samples were obtained from patients suspected of having pneumonia or interstitial lung diseases. Bronchoscopy with BAL and laboratory processing of the recovered BALF have been described previously[7]. Cytocentrifugation was performed with a Shandon Cytospin 3 (Shandon Scientific Ltd., Astmoor, England) using white filter cards (Shandon). In order to obtain monolayer preparations, the number of drops was adjusted to the BALF total cell count[8]. Cytocentrifugation conditions were as follows: speed, 650 revolutions per minute; duration, 10 minutes; acceleration rate, low. Each sample was cytocentrifuged on uncoated, cleaned glass slides (Menzel-Gläser, Braunschweig, Germany) and on PLC slides (Menzel-Gläser) (2 slides each).

Cell density, differential cell counts and cytomorphology

Cell density on the cytocentrifuge spot was assessed by counting the number of nucleated cells on 4 prefixed focal areas in each quadrant of the cytocentrifuge spot using a 10:1 objective. The sum of the areas counted represented a surface of 0.59 mm2, and the number of cells counted was multiplied by 47.84 to express cell density per square millimetre on the cytocentrifuge spot. BALF samples with representative numbers of alveolar

macrophages (Ams), lymphocytes (Lyms) and polymorphonuclear neutrophils (PMNs) were selected for analysis. Freshly spun BALF samples were air dried, and slides were simultaneously stained according to May-Grünwald-Giemsa. Differential cell counts were made on both PLC and uncoated slides (2 each) by 1 observer counting 500 nucleated cells in a circular pattern round the cytocentrifuge spot[7]. Counts were recorded after 100, 200, 300, 400 and 500 cells were enumerated. Apart from AMs, Lyms and PMNs, cell types included were eosinophils (Eos) and mast cells (MCs). Cytomorphology was assessed by a trained observer scoring blindly, on a 500-cell count, the number of free and pyknotic nuclei and the number of cells with vacuolization of the cytoplasm and tearing or stretching of the cell borders.

Statistical analysis

To assess the reproducibility of counting each cell type, variance components were estimated to be used in calculating intraclass correlation coefficients for reproducibility. For each type of slide and cell, “hundreds of cells counted” and “try” were seen as random factors with, respectively, five and two categories. Formulas were based upon Norman’s quasiclassical R coefficients, which are very closely related to the ρ^2-coefficients in generalizability theory[9,10]. From these components the Φ value was calculated. This Φ value can vary from 0 (no reliability at all) to 1 (perfect reliability) and can be used as a reproducibility measure. A Φ value of $\geq$0.95 in counting was considered acceptable[11]. Interslide differences in differential cell counting, cell density and cytomorphology were analyzed by repeated measurements mixed model ANOVA. To overcome problems with negative sums of variance components in Quasi F ratio denominators, Saterthwaite’s and Cockran’s formulas were used to test for statistical significance[12]. All data were analyzed by SPSS-PC, version 10.0.5 (Chicago, Illinois, U.S.A.), and also by GENOVA (University of Massachusetts, Boston, Massachusetts, U.S.A.), a program for generalized analysis of variance[13]. A p-value <0.05 was considered statistically significant.

Assessing clinical relevance

In order to assess clinical relevance, a validated computer program was used to predict the diagnosis in all patients included in the study. The computer program is based on a polychotomous regression model[1] and predicts the patient’s diagnosis using several variables: volume of recovered BALF, total cell count, percentages of AMs, Lyms, PMNs and Eos, and demographic data. The predicted diagnoses generated by the computer program include idiophathic pulmonary fibrosis, sarcoidosis and extrinsic allergic alveolitis.

Results

A total of 20 BALF samples were included for analysis. Although not statistically significant, the cell density on the cytocentrifuge spot was lower on PLC slides as compared to uncoated slides (mean value ± SD: 918.7 ± 595.4 versus 939.9 ± 557.9 cells/mm^2, respectively).
Table 2.1 lists the differential cell counts of PLC slides versus uncoated slides. The PLC slides tended to hold more AMs and significantly fewer Lyms and MCs as compared to the uncoated slides. In 17 of 20 samples, fewer Lyms were counted on the PLC slides as compared to the uncoated slides. The mean value ± SD difference in lymphocyte counts between PLC and uncoated slides was 2.99% ± 3.38% (range 0.10-12.10); in 6 samples, differences >5% were observed. Ams, Lyms, PMNs and Eos counting displayed excellent Φ values on both PLC and uncoated slides. MCs did not achieve the acceptable Φ value on either type of slide (Φ values 0.82 and 0.85 for PLC and uncoated slides, respectively).

Table 2.1 Differential cell counts obtained in 20 BALF samples.

Cell type	PLC slides	Uncoated slides	p-value
Alveolar macrophages	59.43 ± 33.25	57.04 ± 34.10	0.0554
Polymorphonuclear neutrophils	10.89 ± 20.36	10.84 ± 20.92	N.S.
Lymphocytes	25.89 ± 28.26	28.34 ± 29.96	0.0388
Eosinophils	3.38 ± 6.7	3.37 ± 6.54	N.S.
Mast cells	2.03 ± 2.47	2.08 ± 2.47	0.0031

A differential cell count was done by one observer on two slides of both PLC and uncoated slides. For each cell type, mean values ± SD are expressed as a percentage of the 2 × 500 cells counted.

Table 2.2 summarizes the cytomorphologic characteristics. Free nuclei were ore frequently observed on uncoated slides, whereas tearing and stretching of the cell borders were seen more frequently on PLC slides; however, these differences were not statistically significant. The most probable diagnoses predicted by the computer program were as follows: sarcoidosis (n=8), idiopathic pulmonary fibrosis (n=8), extrinsic allergic alveolitis (n=3) and bacterial infection (n=1). There was no difference in the predicted diagnosis between PLC and uncoated slides.

Table 2.2 Cytomorphology of cytocentrifuged BALF samples for both type of slides.

Cell type	PLC slides	Uncoated slides	P value
Free nuclei	13.15 ± 9.39	17.25 ± 14.07	N.S.
Necrobiotic PMNs	2.85 (± 7.21)	3.05 (± 7.13)	N.S.
Tearing and stretching	74.35 (± 76.26)	51.25 (± 57.01)	N.S.
Vacuolated cytoplasma	10.70 (± 12.27)	11.6 (± 14.93)	N.S.

Mean ± SD numbers of free nuclei, necrobiotic PMNs, torn of stretched cell borders and vacuolated cytoplasm are expressed as percentages of 500-cell count.

Discussion

In the present study, we demonstrated that BALF specimens cytocentrifuged on PLC slides tended to hold fewer cells as compared to uncoated slides. In addition, samples cytocentrifuged on PLC slides contained more AMs and significantly fewer Lyms and MCs than those cytocentrifuged on uncoated slides. Concerning cytomorphology, no significant differences were found between samples cytocentrifuged on PLC versus uncoated slides.

As the adhesion of cells to standard microscope slides is low, coating of slide surfaces has been used to enable cells to become more firmly attached and to increase cell yield. Apart from such proteins as albumin and gelatin, various polycations have been evaluated[14]. Polycationic molecules, such as polylysine, are absorbed strongly by solid surfaces, leaving cationic sites that combine with the anionic sites on cell surfaces[4].

With regard to cell adhesion rates, PLC slides have been demonstrated to be superior not only to uncoated slides but also to albumin- and gelatin-coated slides[5,6]. For this reason, our finding of a lower adhesion rate for PLC slides was unexpected. From Table I it is clear that this difference is attributable to the lower recovery rate of Lyms on PLC slides. The mean numbers of AMs were higher on PLC slides than on uncoated slides, and BALF samples with an excess of AMs showed higher total cell recovery on PLC slides (results not shown). AMs are large cells that are known to be more sticky, especially at lower cytocentrifugation speeds[3,15]. By contrast, Lyms in BALF are the cell type most vulnerable to various procedures, including centrifugation and washing of the BALF sample, as well as to the cytocentrifugation process itself[2,16,17]. Indeed, Lyms have been shown to disappear in the filter card during cytocentrifugation[18]. Therefore, we hypothesize that AMs, due to their larger surface as compared to that of Lyms, are relatively more attracted by the cationic surface charges on PLC slides. Lyms, with a smaller surface, are less attracted and, due to their small volume, are prone to disappear into the filter card. The net result is an increase in AMs and relative decrease in Lyms on PLC slides. As, in this study, we used unprocessed BALF samples, we hypothesize that centrifugal and capillary forces during cytocentrifugation

exceed the electrostatic surface charges. This is in line with the finding of previous studies, in which much greater differences in total cell recovery between PLC and uncoated slides were observed when samples were prepared by sedimentation[5] than when they were obtained by cytocentrifugation[6]. With regard to cytomorphology, no significant differences were observed between PLC and uncoated slides. This is in line with the findings of van Oosterbrugge and coworkers[6], who did not find morphologic differences in cytocentrifuged cerebrospinal preparations. In addition, the Φ values for the different cell types were in agreement with previous findings[19]. Although statistically significant, the differences in Lyms counts between PLC and uncoated slides were not clinically relevant in this study. Even in cases of discrepancies >5%, the diagnoses predicted by the computer program were identical for both type of slides.

In conclusion, when compared to that on uncoated slides, the total cell count of BALF samples cytocentrifuged on PLC slides tended to be lower, and PLC slides held fewer Lyms. The present findings do not warrant the routine use of PLC slides for cytocentrifugation of BALF samples.

References

1. Drent M, Jacobs JA, Cobben NA, Costabel U, Wouters EF, Mulder PG. Computer program supporting the diagnostic accuracy of cellular BALF analysis: a new release. Respir Med 2001;95:781-6.
2. Saltini C, Hance AJ, Ferrans VJ, Basset F, Bitterman PB, Crystal RG. Accurate quantification of cells recovered by bronchoalveolar lavage. Am Rev Respir Dis. 1984;130:650-8.
3. De Brauwer EI, Jacobs JA, Nieman F, Bruggeman CA, Wagenaar SS, Drent M. Cytocentrifugation conditions affecting the differential cell count in bronchoalveolar lavage fluid. Anal Quant Cytol Histol. 2000;22:416-22.
4. Mazia D, Schatten G, Sale W. Adhesion of cells to surfaces coated with polylysine. Applications to electron microscopy. J Cell Biol. 1975;66:198-200.
5. Husain OA, Millett JA, Grainger JM. Use of polylysine-coated slides in preparation of cell samples for diagnostic cytology with special reference to urine sample. J Clin Pathol. 1980;33:309-11.
6. van Oostenbrugge RJ, Arends JW, Buchholtz R, Twijnstra A. Cytology of cerebrospinal fluid. Are polylysine-coated slides useful? Acta Cytol. 1997;41:1510-2.
7. De Brauwer EI, Drent M, Mulder PG, Bruggeman CA, Wagenaar SS, Jacobs JA. Differential cell analysis of cytocentrifuged bronchoalveolar fluid samples affected by the area counted. Anal Quant Cytol Histol. 2000;22:143-9.
8. Jacobs JA, De Brauwer E. BAL fluid cytology in the assessment of infectious lung disease. Hosp Med 1999;60:550-5.
9. Shavelson RJ, Webb NM. Generalizability Theroy: A primer. Newbury Park, California: Sage Publications; 1991.
10. Streiner DL, Norman GR. Health measurement scales: a practical guide to their development and use. Oxford: Oxford University Press; 1991.
11. De Brauwer E, Jacobs J, Nieman F, Bruggeman C, Drent M. Test characteristics of acridine orange, Gram, and May-Grunwald-Giemsa stains for enumeration of intracellular organisms in bronchoalveolar lavage fluid. J Clin Microbiol 1999;37:427-9.
12. Kirk RE. Experimental design. Pacific Grove, California: Brooks/Cole Publishing Company; 1982.
13. Crick GE, Brennan RL. GENOVA:a generalized analysis of variance system: Fortran IV computer program and manual. Dorchester, Massachusetts: University of Massachusetts at Boston Computer Facilities; 1982.
14. Seyfert S, Voigt A, Kabbeck-Kupijai D. Adhesion of leucocytes to microscope slides as influenced by electrostatic interaction. Biomaterials. 1995;16:201-7.
15. Willcox M, Kervitsky A, Watters LC, King TE, Jr. Quantification of cells recovered by bronchoalveolar lavage. Comparison of cytocentrifuge preparations with the filter method. Am Rev Respir Dis. 1988;138:74-80.
16. Lam S, LeRiche JC, Kijek K. Effect of filtration and concentration on the composition of bronchoalveolar lavage fluid. Chest. 1985;87:740-2.
17. Mordelet-Dambrine M, Arnoux A, Stanislas-Leguern G, Sandron D, Chretien J, Huchon G. Processing of lung lavage fluid causes variability in bronchoalveolar cell count. Am Rev Respir Dis. 1984;130:305-6.
18. Laviolette M, Carreau M, Coulombe R. Bronchoalveolar lavage cell differential on microscope glass cover. A simple and accurate technique. Am Rev Respir Dis. 1988;138:451-7.
19. De Brauwer EI, Jacobs JA, Nieman F, Bruggeman CA, Drent M. Bronchoalveolar lavage fluid differential cell count. How many cells should be counted? Anal Quant Cytol Histol. 2002; 24:337-41.