Trivariate Flow Cytometric Analysis of Paraffin-Embedded Lung Cancer Specimens: Application of Cytokeratin Subtype Specific Antibodies to Distinguish Between Differentiation Pathways

Mathie P.G. Leers, Paul H.M.H. Theunissen,* Johan Koudstaal, Bert Schutte, and Frans C.S. Ramaekers

1Department of Pathology, De Wever Hospital, Heerlen, The Netherlands
2Department of Molecular Cell Biology and Genetics, University of Limburg, Maastricht (B.S.F.C.S.R.), The Netherlands

Received 16 February 1996; Accepted 30 July 1996

The aim of the present study was to investigate whether trivariate FCM analysis, for the simultaneous detection of two different CK subtypes in combination with DNA content, can be applied to paraffin embedded samples of different types of non-small cell lung cancer in order to evaluate the cell cycle of individual sublines. Single cell suspensions were prepared from 50 μm thick paraffin sections of 22 lung carcinomas by pepsin digestion and immunostained with CK-antibodies which were chosen to distinguish glandular differentiation (adenocarcinomas) and squamous differentiation. There was a good correlation between the immunocytochemical results of the different CK antibodies in tissue sections and in the corresponding single cell suspensions. Gating for CK-positivity revealed a higher S-phase fraction as compared to the ungated cell population. The tumor cells in adenocarcinoma cases were specifically recognized by CK7 antibodies, while well-differentiated squamous cell carcinomas were specifically stained for CK14 and/or CK17. In poorly differentiated squamous cell carcinomas simultaneous expression of CK7 and CK17 was detected in a subpopulation of the tumor cells, next to cells positive for CK7 or CK17 alone. The trivariate FCM analysis allowed the separate estimation of ploidy status and cell cycle parameters in the three different cell populations of these, apparently (phenotypically) heterogeneous, malignancies. Cytometry 27:179–188, 1997. © 1997 Wiley-Liss, Inc.

Key terms: cell cycle analysis; multiparameter analysis; immunohistochemistry

Flow cytometric (FCM) analysis of solid tumors is frequently hampered by the admixture with a variable amount of stromal and inflammatory cells as well as with normal epithelium. By applying the bivariate FCM analysis, combining cytokeratin (CK) immunocytochemistry and quantitative DNA staining procedures, it is possible to study selectively epithelial (cancer) cells (6,29). In a recent study (11) we applied a bivariate DNA analysis to paraffin embedded tissue samples of colorectal carcinomas by using a modification of the enzymatic digestion technique of Hedley et al. (8). It was shown that CK-filaments are retained in the epithelial cells after enzymatic digestion of paraffin embedded tissue (11). Recently, Nylander et al. (15) described a similar method for applying dual parameter analysis to paraffin embedded samples of squamous cell carcinomas of the head and neck region.

In the former study (11) we have shown that the reactivity of a broad panel of antibodies is retained after pepsin digestion. The aim of the present study was to investigate the feasibility of a trivariate FCM analysis of paraffin embedded lung cancers, some of which were heterogeneous in nature, combining glandular and squamous differentiation. Since different types of epithelia and carcinomas contain different types of CKs, the use of monoclonal antibodies to CKs allows the immunohistochemical distinction between different epithelial lineages (19). From previous studies it is known that squamous cell

*Correspondence to: P.H.M.H. Theunissen, Dpt. of Pathology, De Wever Hospital Heerlen, P.O. Box 4446, 6401 CX Heerlen, The Netherlands.
cancers commonly express CKs 14, 15, and 17 (19,23,24), while adenocarcinomas are mainly characterized by CKs 7, 8, 18, 19, or 20 (17,19). For example, extensive CK7 expression in pulmonary adenocarcinomas can distinguish these tumors from squamous cell carcinomas (12). Combining two different monoclonal CK antibodies, specific for either glandular or squamous differentiation, with DNA staining in one assay, would allow a detailed analysis of tumor cell subpopulations in these heterogeneous lung cancers. Such a method, although it might not immediately improve the diagnosis of lung cancer, maybe of general value for the study of processes involved in tumorigenesis, or tumor progression, particularly the accumulation of genomic changes resulting in tumor cell heterogeneity.

MATERIALS AND METHODS

Tumor Samples
Fresh tissue specimens of 22 pulmonary carcinomas were divided into two. One portion was snap frozen in liquid nitrogen and stored at −70°C, and another portion of the same tumor was immediately fixed in 10% buffered formalin for exactly 24 h and routinely processed for paraffin embedding. All lung tumors were classified and graded independently by two pathologists (PT and JK) according to the WHO classification (28).

Immunohistochemistry of Tissue Sections
The panel of monoclonal antibodies (MoAbs) used in this study, and recognizing individual cytokeratins (CKs), is described in Table 1. In order to evaluate the cytokeratin expression in the individual tumors, consecutive 5 µm thick paraffin embedded sections were stained with the different cytokeratin-antibodies as described before (11), by using the streptavidin-biotin-peroxidase complex method and a 3,3’-diaminobenzidine (DAB)- 0.002%H2O2 solution (Sigma Chemical Co., St. Louis, MO) to visualize the immune reaction. Sections were counterstained with Harris’ hematoxylin, dehydrated, cleared in xylene, and finally embedded in Entellan. Simultaneous expression of different CK-subtypes was histomorphologically examined by an immunoenzyme double staining procedure of tissue sections. These double staining experiments were performed by two successive, indirect immunochemical techniques. After incubation with the first MoAb for 1 h at room temperature (RT), this binding was labeled with an indirect streptavidin-biotin-Horse Radish Peroxidase (HRP) method using biotinylated sheep-anti-mouse Ig serum (1:400, Amersham Int., Buckinghamshire UK; 1 h at RT) as bridging antibody, followed by preformed streptavidin-biotin-HRP-complex using an strepABC staining kit (DAKO; 1:1:100; 1 h at RT). Bound HRP was made visible with a 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO)-0.002%H2O2 solution (7). After washing thoroughly with phosphate-buffered saline (PBS), the sections were incubated with the second primary mouse MoAb (1 h at RT). Subsequently, incubation with rabbit anti-mouse Ig (DAKO; 1:50 for 1 h at RT) and monoclonal alkaline phosphatase anti-alkaline phosphatase (APAAP; DAKO, 1:1:100; 1 hr at RT) was performed after appropriate washing. Alkaline phosphatase (AP) activity was detected according to Hoeltke et al. (9); the incubation medium contained naphthol-AS-MX-phosphate (0.2 mg/ml) as substrate and Fast Blue BB (1 mg/ml) as azodye (30 min, 37°C). To inhibit the endogenous AP activity, levamisole was added to the incubation medium to a final concentration of 1 mM.

Immunocytochemistry for Flow Cytometric (FCM) Analysis
The method used for the preparation of single cell suspensions from paraffin embedded tissue has been fully described before (11,21). First, bivariate FCM analysis was performed on 22 lung tumors. In a second series a three-parameter FCM analysis was carried out on 10 selected cases, i.e., tumors showing simultaneous expression of CK7 and CK14 and/or CK17. The indirect immunofluorescence technique was used in combination with primary mouse MoAbs and fluorescein isothiocyanate (FITC) or r-phycoerythrin (RPE)-conjugated secondary immunoreagents. Approximately 106 cells were incubated in 100 µl TRIS buffered saline (PBS; pH = 7.4) containing appropriately diluted primary MoAb (for characteristics and references see Table 1). For triple staining, both primary MoAbs were added simultaneously, i.e., anti-CK7 (a IgG3-mouse MoAb) in combination with anti-CK14 or -CK17 (both IgG2-mouse MoAbs). After an overnight incubation at room temperature, the cells were rinsed twice in TBS. In the bivariate FCM analysis antibody-binding was visualized by incubating the cell pellet, resuspended in...
100 µl TBS, with 6 µl of FITC-conjugated goat-anti-mouse Ig (Fab-fragment; DAKO A/S, Glostrup, Denmark; final dilution 1:20). For triple staining, both secondary antibodies were added simultaneously (i.e., FITC-conjugated goat-anti-mouse IgG1 (1:30) and RPE-conjugated goat-anti-mouse IgG2b (1:30), both from Southern Biotechnology Association, Inc., Birmingham, AL). After incubation in the dark for 11/2 h at room temperature, samples were rinsed twice in TBS and the cells were finally counterstained with propidium iodide (PI; 5 µg/ml, Sigma Chemical Co., St. Louis, MO) in PBS containing 0.1 mg/ml RNase A (Serva, Heidelberg, Germany). The samples were allowed to stand for 15 min on ice in the dark before FCM analysis. For three parameter FCM analysis, the PI concentration was decreased to 1 µg/ml (21). Ten microliters of the cell suspension was examined by fluorescence microscopy to assess the efficiency of immunostaining and the degree of cell clumping. In all samples analysed, doublets comprised less than 5% of the cells studied. As a negative control for CK staining, a cell suspension prepared from a normal lymph node, fixed for 24 h in 10% neutral buffered formalin and embedded in paraffin, was used. As a negative control for background staining, lung cancer cells were stained using the mouse anti-BrdUMoAb (clone IIB5) (20). To control for bleed through of the different fluorochrome signals, single FITC, RPE, or PI stained samples were FCM analysed. To control for cross reactivity of the Ig-isotype-specific secondary antibodies, the appropriate primary antibody was omitted from the immunocytochemical double-staining procedure. For all three-parameter FCM analyses no cross reactivity of the secondary antibodies was observed (data not shown).

**Flow Cytometry**

All samples were analysed using a FACScan flow cytometer (Becton and Dickinson, San Jose, CA). Fluorochromes were excited at 488 nm by an Argon laser. FITC-fluorescence was detected through a 515–545 nm BP filter, RPE-fluorescence through a 572–588 nm BP filter, and PI-fluorescence through a 600 nm LP filter. FITC and RPE signals were recorded as logarithmic amplified data, while the PI signals were recorded as linear amplified data. For bivariate FITC/PI analysis no compensation was used. In the tricolor analyses, cross talk between the different channels was compensated using the following settings: 448, 405, and 437 V on photomultiplier tubes for FL1 (FITC), FL2 (RPE), and FL3 (PI), respectively. Compensation for (FL1-%FL2), (FL2-%FL1), (FL2-%FL3), and (FL3-%FL2), was 11%, 38%, 41%, and 8.3%, respectively. Cell cycle analysis was performed with CellFit software (Becton and Dickinson, San Jose, CA). The S-phase fraction (SPF) was calculated from histograms of ungated cells and from cells gated for CK-positivity. For both DNA-diploid as well as DNA-aneuploid tumors, the polynomial model (18) was used. SPF calculation was only performed when the CK-positive gate comprised at least 4,000 events. A tumor sample was designated positive for a specific CK subtype when the percentage of CK-positive cells (designated as CK-positive cell fraction) comprised more than 5% of the whole cell suspension.

**RESULTS**

When 50 µm thick deparaffinized and rehydrated tumor tissue sections were digested by pepsin for 80 min (37°C), a sufficient number of cells could be obtained to perform bivariate FCM analyses. When examined in the fluorescence microscope it became evident that these isolated cells had retained enough cytoskeletal remnants around the nucleus to allow detection of several CK-subtypes (11).

**Cytokeratin Subtype Immunoreactivity: Paraffin Tissue Sections vs. Cell Suspensions (Table 2)**

First of all, we examined the extent of agreement between the immunochemical results with the MoAbs in tissue sections and in the cell suspensions of the corresponding paraffin embedded tumors. A broad panel of MoAbs against CKs was tested (Table 1). The series of lung carcinomas consisted of 22 cases: 10 squamous cell carcinomas, 5 adenocarcinomas, 5 large cell undifferentiated carcinomas, and 2 adenosquamous carcinomas. The specificity of the different CK-MoAbs in the lung cancer cell suspensions is summarized in Table 2. The results obtained in tissue sections correlated well with the FCM results obtained from cell suspensions for all six antibodies.

**Squamous cell carcinoma.** An extensive CK-positive cell fraction for CK14, CK17, and CK19 was present in the majority of the samples, both tissue sections and cell suspensions. CK7-expression was observed in about half of the squamous cell carcinomas, mainly in the poorly differentiated cases. This phenomenon was also observed for the expression of CK8 (in tissue sections as well as in cell suspensions). In cell suspensions the greatest CK-positive cell fraction was obtained with the CK17- and CK19-antibodies.

**Adenocarcinoma.** These tumors were in all cases positive for CK7, CK8, and CK19, both in tissue sections as well as in cell suspensions. CK17 was found in a single case, with only a small CK positive cell fraction.

**Large cell anaplastic carcinoma.** These tumors showed in almost all cases clear expression for CKs 7, 14, and 19, both in tissue sections as well as in cell suspensions. Three out of five samples were positive for CK17. CK7-immunoreactivity was found in the largest tumor cell fraction of these cell suspensions.

**Adenosquamous carcinoma.** The two cases of adenosquamous carcinoma investigated were both positive for CK7, CK8, and CK19. One tumor was also positive for CK14 and CK17. This simultaneous expression was also seen in the cell suspension of that tumor. Again, the greatest CK positive cell fraction was obtained with the CK7-antibody.
The next step was to examine the feasibility of the CK/DNA bivariate FCM analysis in cell suspensions derived from paraffin blocks. For this purpose, the squamous cell carcinomas (n = 10) and adenocarcinomas (n = 5) were analysed. From all these 15 cases both freshly frozen tissue as well as formalin-fixed paraffin embedded tissue samples were available. The results with the MoAbs against CKs 7, 8, 19, and 20 are comparable to those of our previous study on colorectal carcinomas (11) and can be summarized as follows (see Table 3):

- the staining results in tissue sections with the different antibodies correlated well with the staining results of the cell suspension prepared for flow cytometry;
- the immunoreactivity patterns for the different CKs were similar when suspensions from paraffin and frozen tissues of the individual cases were compared;
- the CK-positive fractions were considerably higher in cell suspensions derived from fresh frozen tissue as compared to the corresponding paraffin sample;
- a good correlation was found between the DNA-index obtained from cell suspensions prepared from either fresh frozen or formalin-fixed, paraffin-embedded tumor samples;
- in comparison to the ungated cell population, the SPF values were in general increased when this analysis was performed after gating for CK-positivity, both in paraffin and fresh frozen tissue samples. This phenomenon was more pronounced for paraffin embedded samples.

**Cell Cycle Analysis of CK Positive Cells**

The cell suspensions prepared from the 22 cases of paraffin embedded lung carcinomas were also further examined for cell cycle parameters by FCM (see Table 2). The CV of the G0/G1-peak of the tumor cells was within acceptable limits (22) (mean CV G0/G1 peak 3.4–5.3%) and decreased in the majority of the cases after gating for CK-positivity. For all these cases of lung carcinomas, the SPF was increased after selectively analyzing the CK-positive cells (Table 2). Striking differences in SPF were observed when cell fractions gated for the various CK subtypes were compared, suggesting heterogeneity within the tumor tissue.

**Three-Parameter FCM Results of Paraffin Embedded Lung Carcinomas (Table 4)**

To investigate the feasibility of the trivariate FCM protocol for the analysis of heterogeneity of paraffin embedded lung cancer, ten carcinomas (five squamous cell carcinomas, three large cell carcinomas, and two...
adenocarcinomas) were selected based on their simultaneous expression patterns in tissue sections of CKs typical for glandular differentiation (CK7, CK8, and CK19) and CKs of squamous differentiation (CK14 or CK17). These ten carcinoma samples were analysed by three-parameter FCM for DNA content and the combined expression of either CK7 and CK14 or CK7 and CK17.

The five carcinomas positive for CK14 comprised four squamous cell carcinomas (cases 1, 8, 16, and 26) and one adenocarcinoma (case 23) as summarized in Table 4. In cases 1 and 8, the aneuploid cells exclusively express CK14, while the diploid cells were CK7 positive and CK14 negative. Microscopic analysis of the tissue section revealed CK7 positivity only in normal type II pneumocytes (see Fig. 1A). When gating for these CK7 positive cells only a diploid cell population was indeed found. In the other two squamous cell carcinoma samples (cases 16 and 26, Table 4), the CK14 expression patterns were more heterogeneous (see Fig. 1B), with aneuploid cells in both the CK7+/CK14- and the CK7+/CK14+ compartments as shown after gating. In the CK14 positive adenocarcinoma sample (case 23) the aneuploid fraction was limited to the CK7+/CK14- population.

The nine carcinomas positive for CK17 comprised five squamous cell carcinomas (case 1, 8, 16, 19, and 26), one adenocarcinoma (case 11) and three large cell anaplastic carcinomas (cases 6, 22, and 21). Again, in cases 1 and 8 the CK7 positive (DNA diploid) cell fraction was negative for CK17 (see Fig. 1A). On the contrary, the CK17 positive tumor cells of this case showed a predominant DNA aneuploid stemline. In the other CK17 positive cases, except for case 21, the CK expression patterns were more

---

### Table 3

Comparison of Bivariate FCM Results Obtained From Suspensions Derived From Fresh Frozen and From Paraffin Embedded Tumor Samples (n=15)

<table>
<thead>
<tr>
<th></th>
<th>UG</th>
<th>CK 7⁺</th>
<th>CK 8</th>
<th>CK 19</th>
<th>CK 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous cell carcinoma (n=10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of positive cases:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh frozen</td>
<td>—</td>
<td>3/10 b</td>
<td>6/10</td>
<td>9/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Paraffin</td>
<td>—</td>
<td>5/10</td>
<td>5/10</td>
<td>8/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Mean CK pos. cell fraction (%)</td>
<td>—</td>
<td>32</td>
<td>31</td>
<td>39</td>
<td>12</td>
</tr>
<tr>
<td>Fresh frozen</td>
<td>—</td>
<td>14</td>
<td>14</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>Paraffin</td>
<td>—</td>
<td>14</td>
<td>14</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>Mean SPF (%)</td>
<td>—</td>
<td>13 (7-19)²</td>
<td>18 (9-27)</td>
<td>12 (4-20)</td>
<td>21 (12-30)</td>
</tr>
<tr>
<td>Fresh frozen</td>
<td>—</td>
<td>13 (6-20)</td>
<td>21 (19-23)</td>
<td>32 (24-40)</td>
<td>28 (22-34)</td>
</tr>
<tr>
<td>Paraffin</td>
<td>—</td>
<td>13 (6-20)</td>
<td>21 (19-23)</td>
<td>32 (24-40)</td>
<td>28 (22-34)</td>
</tr>
<tr>
<td>Adenocarcinoma (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of positive cases:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh frozen</td>
<td>—</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Paraffin</td>
<td>—</td>
<td>4/5</td>
<td>5/5</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Mean CK pos. cell fraction (%)</td>
<td>—</td>
<td>28</td>
<td>33</td>
<td>41</td>
<td>12</td>
</tr>
<tr>
<td>Fresh frozen</td>
<td>—</td>
<td>38</td>
<td>18</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>Paraffin</td>
<td>—</td>
<td>38</td>
<td>18</td>
<td>28</td>
<td>3</td>
</tr>
<tr>
<td>Mean SPF (%)</td>
<td>—</td>
<td>13 (3-23)</td>
<td>17 (7-27)</td>
<td>21 (16-26)</td>
<td>16 (6-26)</td>
</tr>
<tr>
<td>Fresh frozen</td>
<td>—</td>
<td>11 (7-15)</td>
<td>23 (14-32)</td>
<td>33 (29-37)</td>
<td>30 (22-38)</td>
</tr>
<tr>
<td>Paraffin</td>
<td>—</td>
<td>11 (7-15)</td>
<td>23 (14-32)</td>
<td>33 (29-37)</td>
<td>30 (22-38)</td>
</tr>
</tbody>
</table>

*²Number of positive cases over number of cases tested.

*Between brackets: range of individual cases SPF, S-phase fraction; UG, un gated, no label.

### Table 4

DNA-Index of the Different Epithelial Lineages Within One Tumor, as Defined by their CK Expression Pattern

<table>
<thead>
<tr>
<th>Case nr.</th>
<th>Tumor type</th>
<th>Total cell pop.</th>
<th>CK7⁻/ CK14+</th>
<th>CK7⁺/ CK14⁻</th>
<th>CK7⁻/ CK14+</th>
<th>CK7⁻/ CK17⁻</th>
<th>CK7⁺/ CK17⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>SQC, G1</td>
<td>1.00-1.57</td>
<td>1.56</td>
<td>1.00</td>
<td>—</td>
<td>1.55</td>
<td>1.00</td>
</tr>
<tr>
<td>8a</td>
<td>SQC, G1</td>
<td>1.00-1.64</td>
<td>1.59</td>
<td>1.00</td>
<td>—</td>
<td>1.50</td>
<td>1.00</td>
</tr>
<tr>
<td>16</td>
<td>SQC, G3</td>
<td>1.00-1.49</td>
<td>—</td>
<td>1.00-1.50</td>
<td>—</td>
<td>1.00-1.49</td>
<td>1.00</td>
</tr>
<tr>
<td>19</td>
<td>SQC, G3</td>
<td>1.00-1.49</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.00</td>
<td>1.00-1.51</td>
</tr>
<tr>
<td>26</td>
<td>SQC, G3</td>
<td>1.00-1.84-2.08</td>
<td>1.00-1.88</td>
<td>1.00-1.83</td>
<td>—</td>
<td>1.00-1.84-2.13</td>
<td>1.00-1.85-2.08</td>
</tr>
<tr>
<td>11</td>
<td>AC, G3</td>
<td>1.00-2.02-2.73</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.00-2.01-2.68</td>
</tr>
<tr>
<td>23</td>
<td>AC, G3</td>
<td>1.00-1.60</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00-1.58</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>LCAC, G4</td>
<td>1.00-2.16</td>
<td>—</td>
<td>—</td>
<td>1.00-2.17</td>
<td>1.00-2.16</td>
<td>—</td>
</tr>
<tr>
<td>22</td>
<td>LCAC, G4</td>
<td>1.00-1.63-2.18</td>
<td>—</td>
<td>—</td>
<td>1.00-1.64-2.21</td>
<td>1.00-1.63</td>
<td>1.00-1.63</td>
</tr>
<tr>
<td>21</td>
<td>LCAC, G4</td>
<td>1.00</td>
<td>—</td>
<td>—</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*²Cases of single-cell suspensions containing normal diploid (CK 7 positive) epithelial cells.

*The number of cells was too small to calculate a reliable DNA-index. SQC, squamous cell carcinoma; AC, adenocarcinoma; LCAC, large cell anaplastic carcinoma; CK, cytokeratin; G1, well-differentiated; G3, poorly differentiated; G4, undifferentiated.
FIG. 1. (Legend on page 186).
FIG. 1. (Legend on page 186)
heterogeneous, showing aneuploid cells in various CK subpopulations (see Fig. 1C).

In some cases a substantial enrichment of the aneuploid fraction could be achieved by gating on various combinations of CK expression patterns (see Table 4, cases 1, 8, 23, and 26, and Fig. 1B), while in others the relative frequency of aneuploid cells remained unchanged after gating as compared to the ungated data (see Fig. 1C).

**DISCUSSION**

In a recent study, a method for the bivariate CK/DNA flow cytometric analysis of paraffin embedded samples of colorectal carcinomas was developed (11). It became apparent that the nuclei present in the suspensions obtained from the paraffin embedded tissues had retained enough CKs to allow their FCM recognition as epithelial cells. The aim of the present study was to further develop this method in such a way that different lineages of epithelial differentiation, present within a (heterogeneous) tumor sample, could be analysed separately by FCM. For this purpose we have selected lung cancer cases, which are known to often present two or more types of differentiation within the same tumor. Based on the findings of our earlier study (11), we have selected antibodies that give similar results, in particular with respect to the percentage of immunoreactive cells, when comparing paraffin embedded tissue sections and cell suspensions derived from these paraffin blocks. Monoclonal antibodies against cytokeratin 7 can distinguish pulmonary adenocarcinoma (17,27), while CK14 and CK17 have been associated with squamous differentiation in lung carcinomas (27).

**Bivariate FCM Analysis of Lung Tumors**

The CK expression patterns of the different types of lung carcinomas may show a considerable heterogeneity. First of all, CK7, which is considered to be a marker for glandular differentiation (12) and to be largely absent in squamous cell carcinomas (14), was expressed in a part of the tissue sections as well as in the cell suspensions of five out of ten cases of pulmonary squamous cell carcinoma investigated in this study. In all these five cases it marked poorly differentiated tumors, as described before by Ramakers et al. (16). In squamous cell carcinomas CK7 expression is apparently related to the degree of differentiation, since well-differentiated and moderately differentiated squamous cell carcinomas were virtually all negative for CK7. Second, the results of the present study show that CK14, considered as a marker of squamous epithelium, is expressed in four out of five cases of large cell anaplastic carcinomas. It may however well be possible that some lung carcinomas classified as large cell anaplastic carcinomas would better be classified as poorly differentiated squamous cell carcinomas of the lung on the basis of CK-expression patterns. The histological distinction between a poorly differentiated squamous cell carcinoma and a large cell anaplastic carcinoma is often difficult. In general, most of the antibodies used in this study displayed in the cell suspensions an immunoreactivity pattern similar to that found in the tissue sections. In five cases, however, which were positive in the tissue sections, no immunostaining with any of the CK antibodies was detected. This may be due to technical factors, such as tissue processing or extended enzymatic digestion. The CK14-immunopositive fraction in cell suspension was in general small compared to the extended fraction that could be stained for CK14 in the tissue section. Masking of the CK14-epitope in paraffin embedded material might be an explanation for this phenomenon because for immunohistochemistry on tissue sections an antigen-retrieval method (micro-wave heating) is recommended when using antibody LL002 (25). In the present study, acceptable CVs (3.4 to 5.3%) were measured for the cell suspensions derived from paraffin embedded tumor samples. In general CV values improved after gating for the CK positive fractions, which can be explained by the elimination of necrotic material and stromal debris (4,5).

When samples, double labeled for DNA content on the one hand and the different CK subtypes on the other, were submitted to a detailed cell cycle analysis, it was observed that the SPF values increased after gating on CK positive cells. This could be explained by the selective elimination of the nonepithelial cells, such as inflammatory and stromal cells. Furthermore a general tendency was observed for both adenocarcinomas and squamous cell carcinomas to show the highest mean SPF in the cell fractions expressing CK8, characteristic for simple epithelia, compared to cells expressing more lineage-related Cks, such as CK7 or CK17.
In squamous cell carcinomas, a correlation was found between degree of differentiation and SPF in tumor cell fractions expressing CK 14 or CK 17. In poorly differentiated tumors the cell fractions expressing CK 14 or CK 17 tend to have a higher SPF than the same fraction in well-differentiated tumors. In adenocarcinomas such tendency was observed in cell fractions expressing CK 7 or CK 8.

Three-Parameter FCM Analysis of Lung Tumors

The simultaneous expression of CKs of the simple-epithelial-type (CKs 7, 8, and 19) and those typical of stratified epithelia (CKs 14 and 17), as seen in 10 out of 22 lung carcinomas examined was also observed before by several authors (1–3, 27).

In all the cases that co-expressed these CKs for different epithelial lineages, co-expression marked poorly differentiated lung tumors. Three-parameter FCM revealed that simultaneous expression of CK 7 and CK 14 (or CK 17) in lung tumors is very often due to co-expression of these CKs in most of the tumor cells (seven out of ten cases; compare Fig. 1B). In two cases, the differential expression of the two CK-subtypes was due to an admixture of normal diploid pneumocytes, which are positive for CK 7. In all the seven cases described above, there were also different cell populations each of which was exclusively expressing one specific CK subtype. In a few cases, different clonal stemlines were found within one and the same tumor, all exhibiting a different set of CK-subtypes. This indicates that, within one tumor, mosaics of cells or groups of cells can exist, each expressing a different set of CKs and with a different DNA-index.

In summary, trivariate CK/DNA FCM analysis (simultaneous immunostaining for two different subtype specific CKs as well as DNA) is a technical advance for a reliable cell-kinetic analysis of human tumor cell populations. The immunoreactivity for the different CK-subtypes is well preserved in the single cell suspensions prepared from the paraffin embedded tumor samples. Heterogeneous CK-expression can be correlated to different DNA stemlines and also to the cell cycle kinetic parameters of such tumor cell subpopulations. Prospective studies are needed to further evaluate the correlation between degree of differentiation and SPF in tumor cell fractions expressing CK 14 or CK 17 that was observed in squamous cell carcinomas. It is now also possible to investigate DNA patterns in different epithelial cell lineages (each expressing a different set of CKs) within heterogeneous tumors such as lung carcinomas. The use of formalin fixed, paraffin embedded tumor blocks facilitates retrospective studies. In this study, we have used a standardized fixation protocol. However, a potential pitfall associated with the use of archival histological material is the lack of uniformity in tissue fixation. Thus, further study is necessary to investigate if prolonged or delayed fixation influences the above-described results.

LITERATURE CITED


