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Sensitive Three-parameter Flow-cytometric Detection of Abnormal Cells in Human Cervical Cancers: A Pilot Study

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Summary. The cell volume, the DNA, and the carcino-embryonic (CEA) or epithelial-membrane (EMA) antigen of formaldehyde-fixed human cervical cells from 21 malignant cervix tumors and 11 normal patients were measured simultaneously with a Fluvo-Metricell flow cytometer. The simultaneous cell volume and DNA measurement provided the distinction of morphologically intact cells from cell debris, the determination of the cell cycle phase combined with the detection of aneuploid cells, and the distiction of inflammatory cells from parenchymal and tumor cells. Malignant samples were recognized because they contained more than 0.5% CEA positive cells which were of intermediate size. CEA and EMA expression in the malignant samples was not linked. The false positive rate in a total of 32 samples was 6.3% when the sum of CEA and EMA positive cells of each cell sample was calculated. No false negative malignant sample was observed.

Key words: Flow-cytometry – Cervical cancer – Carcino embryonic antigen (CEA) – Epithelial membrane antigen (EMA) – Cell volume

Introduction

The screening for cervical cancers is mostly manually performed by microscopical examination of Papanicolaou stained cell smears (Soost and Baur 1980). The evaluation of many smears as they occur commonly is time-consuming and subject to human errors. Considerable efforts have been made to automate the recognition of malignant cells either by computer-controlled automated image analysis (Al and Ploem 1979; Abmayr 1979) or by flow cytometry (Goerttler and Stöhr 1979; Barrett et al. 1979; Scheiffarth et al. 1979). The time requirement of the computer-evaluated images is still a limiting factor today. Flow cytometry, in contrast, is a faster method allowing 1,000–2,000 cells to be analyzed per second. Flow-cytometric measurements do not normally resolve morphological details of the cells but their advantage is that several defined biochemical of biophysical parameters can be measured simultaneously for each single cell. It is also possible to photograph

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the cells in flow, a technique which provides a combination between flow cytometry and image analysis (Kachel et al. 1979, 1980; Kay et al. 1979). Flow-cytometric methods have been used to identify cervical tumor cells with parameters, such as DNA, protein, and light scatter. One-parameter (Sprenger 1979; Leary et al. 1979), two-parameter (Goerttler and Stöhr 1979; Barrett et al. 1979; Habbersett et al. 1979; Scheiffarth et al. 1979), or three-parameter (Stöhr and Futterman 1979) measurements have been performed. Although it was possible to detect typical locations of tumor cells in the flow-cytometric histograms, there has not been sufficient sensitivity for the detection of a few tumor cells amongst many normal cells until now. Typically, between 5% and 20% abnormal cells had to be present to identify the sample as malignant.

The aim of this study was to improve the sensitivity of the flow-cytometyric measurement to detect 0.5% of abnormal cells and to avoid false negative or false positive results as far as possible. This was achieved by simultaneously measuring antigenic determinants on the cell curface together with cell volume and cellular DNA.

Methods

Patients

The mean age of the normal patients was 33.1 years with a standard error of 1.6 years ($N=11$). The youngest patient was 22 and the oldest 42 years old. The cytologic classification was either Pap. I or Pap. II. The tumor samples were taken from the tumor surface prior to treatment. The mean age of the patients was 55.9 ± 13.2 years ($N=21$). The youngest patient was 33 years and the oldest 73 years old.

Cell Preparation

Smears were taken from the cervix uteri with a cotton swab wrapped around a plastic rod. The cotton swab was transferred into a 50 ml plastic centrifuge tube filled with 20 ml 0.15 *M* NaCl, 10 m*M* TRIS/HCl (TBS) buffer, pH 7.4. The centrifuge tube was manually shaken for 1 min to separate the cells from the cotton swab. The cotton swab was removed, and the cells were syringed once through needles of 1.0, 0.8, and 0.65 mm inner diameter to prepare a single cell suspension. The cells were filtered through a steel sieve of a quadratic pore size of 50×50 µm. The cell suspension was washed twice with 10 ml TBS, centrifuged at 800 *g* for 10 min, and resuspended in 3 ml TBS. The cells were fixed by addition of an equal volume of a 3.5% formaldehyde solution in TBS which was freshly prepared and titered shortly prior to fixation to pH 7.4 by addition of 1 N NaOH solution. Between 3 to 8×10^5 cells were obtained from each smear.

Indirect Immunofluorescence

The fixed cells were washed twice in 10 ml TBS with 50 mg/ml bovine serum albumin (BSA) added and resuspended in 50 µl goat-anticarcinoembryonic (CEA) or goat-antiepithelial-membrane-antigen (EMA) serum (Heyderman et al. 1979; Sloane and Ormerod 1981) and incubated for 2 h at 0 °C with occasional shaking. Both antibodies were affinity-purified IgG-preparations and diluted 1/25 in TBS buffer with 50 mg/ml BSA (TBS/BSA) prior to the incubation. The controls contained 1/50 diluted whole-goat serum in TBS/BSA. BSA was added in all instances to decrease the non-specific adherence of immunoglobulins to the fixed cells. The cells were washed twice after this incubation with 1 ml TBS/BSA and reincubated in 50 µl fluorescein-isothiocyanate (FITC)-labeled IgG-fraction of a rabbit anti-goat antiserum (molar F/P ratio = 2.0) in a dilution of 1/25 in TBS/BSA for 2 h at 0 °C. The cells were washed twice with 1 ml TBS/BSA. The pellet was resuspended in 250 µl of a Mithramycin solution in TBS (100 µg/ml with 15 m*M* MgC 12) and incubated for 2 h at 0 °C to stain the cellular DNA (Crissman et al. 1976).

Flow Cytometry

The cell volume, the immunofluorescence of CEA or EMA antigen, and the cellular DNA were simultaneously measured with a Fluvo-Metricell flow cytometer (Kachel et al. 1977). The cell volume was

determined electrically with a hydrodynamically focused cylindrical orifice of 85 µm diameter and 100 µm length using an electrical current of 0.0492 mA. The conductivity of the suspending TBS buffer was 16.5 m Siemens at 22 °C. The cellular fluorescence was excited by epi-illumination with a HBO-100 mercury high pressure lamp between 418 and 500 nm. The FITC-fluorescence was collected between 500–530 nm and the mithramycin fluorescence between 530–600 nm using a 530 nm dichroic mirror (Carl Zeiss, Oberkochem, FRG). The cell volume signals were amplified with a 2.5 decade logarithmic amplifier while both fluorescence signals were linearly amplified. The maximum amplitude of the two fluorescence signals and of the volume pulse of each cell were collected in list mode on magnetic tape. Between 0.5 and 1.5×10^5 particles were measured per sample. The primary data on tape were evaluated graphically and quantitatively by Fortran IV computer programs developed earlier (Valet 1980; Valet et al. 1979). The fluorescein and the mithramycin fluorescence emission spectrum overlap. The overlap of each fluorescence signal of the list mode data was corrected in the following way. Fluorescence signals with an amplitude of 27% (b) of the amplitude in the fluorescein channel were observed in the mithramycin channel when the sample was only stained with fluorescein. The overlap from the mithramycin signals was 58% (a) into the fluorescein channel when the sample was only stained with mithramycin. Knowing the fluorescence overlap for both substances (a, b) and assuming that the fluorescences do not influence each other, the measured fluorescence in the fluorescein channel (x') of a double-stained sample is composed of the fluorescein fluorescence (x) and the fluorescence overlap (a) from the true mithramycin fluorescence (y') in the mithramycin channel: $x' = x + ay$. A similar equation is valid for the measured fluorescence (y') in the mithramycin channel: $y' = y + bx$. Resolution of both equations yields:

1. $x = (x' - by')/(1 - ab)$, and
2. $y = (y' - ax')/(1 - ab)$.

The assumption that the fluorescence signals of both substances do not influence each other in a double stain seems justified because the mithramycin and fluorescein molecules are spatially separated in the cell nucleus and on the cell membrane which makes energy transfer effects unlikely. The result of the calculation can be controlled in a simple way. The G0/G1 cells of cervical smears when stained with mithramycin alone form an elongated cell cluster which is parallel to the cell volume axis in the contour plot. The cluster is not parallel when fluorescence from a double stain overlaps into the mithramycin channel. When the measured fluorescence pulses are now properly corrected for overlap in both fluorescence channels using Eqs. 1 and 2 the G0/G1 cloud of cells is parallel to the cell volume axis (Fig. 1a, b).

Results

The graphical display (Fig. 1a) of a simultaneous three-parameter measurement (cell volume, DNA, CEA-antigen) of normal cervical cells shows that most cells are in the Go/G1 phase of the cell cycle and do not react positively with the CEA-antiserum. There is, however, a significant amount of cellular debris and also of large superficial cells which are positive for the CEA antigen. The antigen-positive debris is well separated from the G0/G1 phase of the morphologically intact cells which is favorable for the subsequent quantitative evaluation. Morphologically intact cells have both a DNA and a cell volume signal. Bare nuclei show only a DNA signal and enucleated cells only a volume signal. The superficial cells are the large cells above volume class 16 (Fig. 1a). They can be well distinguished from the intermediately sized cells where tumor cells occur in smears from cervical tumors. The superficial cells are CEA-antigen-positive (Fig. 1a). This fluorescence can be suppressed by the admixture of 50 mg/ml BSA indicating that the fluorescence was an unspecific fluorescence. BSA was, therefore, routinely admixed for all measurements. Samples from cervical tumors contain CEA-positive cells in the intermediate size range of nucleated cells (Fig. 1b). They are visible as a finger-like protrusion.

The quantitative evaluation of cells of 21 untreated malignant cervical tumors and 11 normal cases shows almost no overlap between the normal and malignant

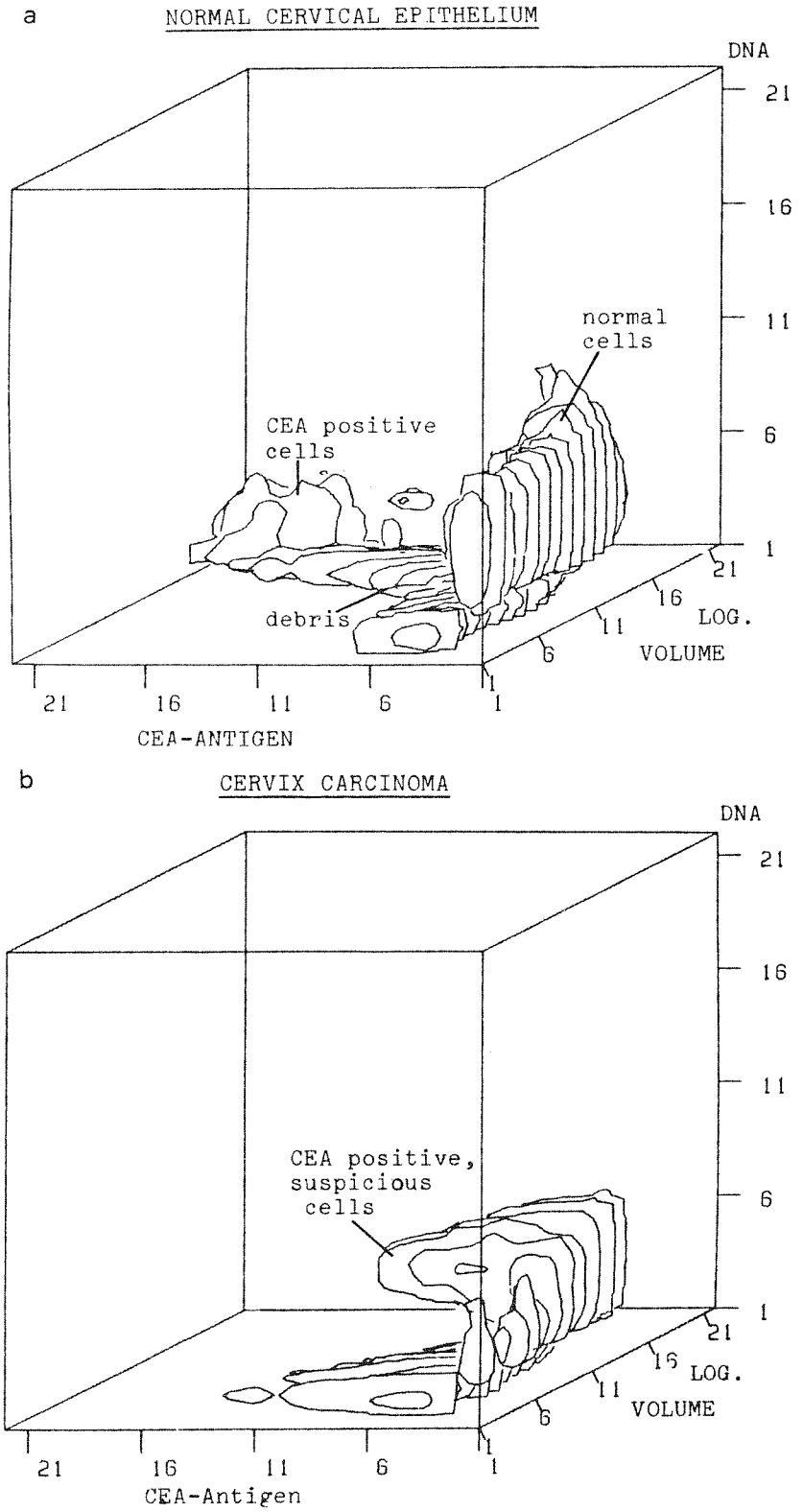

Fig. 1 a, b. a Display of a simultaneous three-parameter measurement (cell volume, DNA, CEA antigen) of a normal sample of human cervical cells (83,726 particles) and **b** of a sample from a cervix tumor (103,089 particles). The contour lines in both panels represent 10% of the maximum amplitude of each histogram. Anti-CEA serum of Hofmann-La Roche (Scheiffarth et al. 1979) was used for both measurements. The cells were incubated in the antiserum without BSA addition in the upper panel, while BSA was added in the assay of the lower panel. The fluorescence of the large superficial cells in the left panel was unspecific because it could be suppressed by BSA (not shown). The immunofluorescence of the cells of intermediate size in the right panel was specific because it remained in the presence of BSA

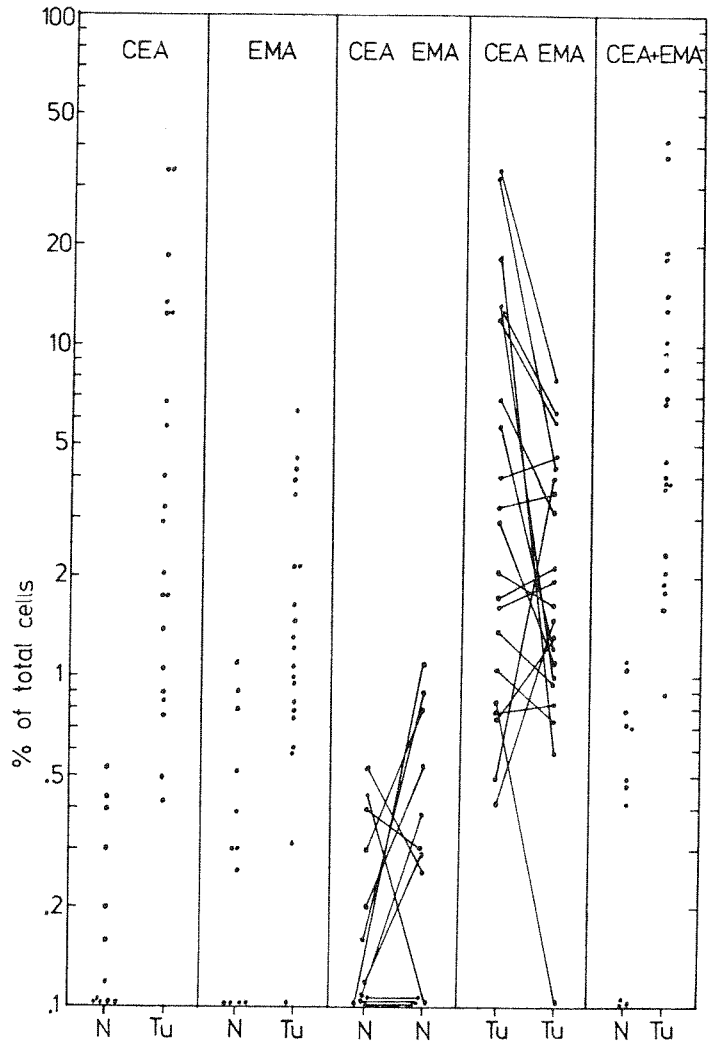

Fig. 2. Expression of CEA and EMA antigens on cells of normal *N* or malignant *Tu* samples of the human cervix uteri

samples (Fig. 2). The samples were randomly chosen from normal patients from a prescreening program and from cervical tumor patients. The malignant samples contain between 0.4 and 37% of CEA-positive cells, while the normals have between 0.05 and 0.5% positive cells. The difference between normal and malignant samples is somewhat less pronounced for the EMA antigen (Fig. 2). The comparison between the CEA and the EMA assay of cells of the same patient shows that the expression of both antigens on the cell surface is not linked because samples with high CEA and low EMA antigenicity and others with high EMA and low CEA are observed (Fig. 2). The ratio between % CEA- and % EMA-positive cells is a quantitative descriptor of these observatoins. Sixty-two per cent of the samples contained more CEA- than EMA-positive cells and 38% less CEA- than EMA-positive cells.

Discussion

The simultaneous three-parameter analysis of the cervical cells according to cell volume, DNA, and cell surface antigens is 10–40 times more sensitive as a one- or simultaneous two-parameter measurement since 0.5% abnormal cells are recognized as compared to 5–20% when light scatter, DNA, or protein are used as parameters. The three-parameter measurement decreases possible sources of error.

The problem of unspecific binding of antibody to cell debris is solved by the simultaneous cell volume and DNA measurement by which morphologically intact cells are easily identified. The DNA determination indicates in addition the cycle phase of each cell and allows the recognition of aneuploid cells. The cell volume indicates whether the abnormal cells occur in the intermediate size range which is typical for tumor cells. Cells smaller than volume class no. 7 (Fig. 1a) are inflammatory lymphocytes or granulocytes, cells greater than volume class no. 16 (Fig. 1a) are superficial cells. An advantage of the antigenic parameters is the decrease of false alarms which are normally due to cell aggregates. They occur if parameters, such as light scatter, DNA, and protein are used in flow cytometry (Cambier and Wheeless 1979; Goerttler and Stöhr 1979). Heavy multicellular aggregates do not occur in our preparation of cervical cells due to the filter step during preparation. Remaining small cell aggregates are not misclassified because aggregates of only normal cells will be classified as normal since no antigenic fluorescence is detected. The aggregate is classified as positive if one or more cells of an aggregate are tumor cells with positive antigenic fluorescence. From this it follows that aggregates alter the quantitative but not the qualitative result of the analysis. The most important point is that no positive event is lost. This is essential for the recognition of small proportions of tumor cells amongst many normal cells.

The DNA distributions in these measurements (Fig. 1a, b) have coefficients of variation (CV) for the G0/G1 peak between 6% and 9%. This is substantially higher than values around 1% as obtained by Göhde et al. (1980) with human sperm cells. There are several reasons for the higher CVs in our study: first, formaldehyde fixation increases the CV of the mithramycin stain as compared to ethanol fixation. Formaldehyde fixation is, however, preferable because the cell volume and the volume distribution curve is better conserved. Second, the mithramycin flurorescence was considerably brighter than the fluorescein immunofluorescence. The mithramycin excitation was, therefore, limited to a range between 418 and 500 nm. The mithramycin and fluorescein signals were then of comparable intensity which is important for the distinction of both signals by the photomultipliers. The inconvenience in the limitation of excitation is that the noise contribution to the mithramycin signal becomes higher which broadens the DNA distributions and increases their CV. Third, the use of low amplitude contour lines (10% of the maximum channel content, Fig. 1a, b) broadens the DNA distribution in the graph artificially. This is not so if 40%, 60% or 80% contour lines are plotted. The CEA- or EMA-positive cells are, however, not displayed at these high contour levels because their frequency is low. The choice of a low-level contour line is, therefore, adequate for the demonstration of abnormal cells, but one has to accept that the DNA distributions appear broadened. The broad DNA distributions make it difficult to distinguish slightly hyperdiploid abnormal cells in the contour graphs (Fig. 1b) from the rest of normal diploid cells. The abnormal cells in most of the tumor samples were located between the diploid and octoploid DNA levels which is consistent with the well known fact that many cervical tumors are aneuploid. The main goal of the simultaneous cell volume and DNA measurement which was to separate the morphologically intact cells from cell debris is reached by our method despite the broad DNA distributions. The compromise that some resolution of the DNA distribution is lost is not a limiting factor because the dis-

tinctive criterium for the identification of the abnormal cells is mainly the positive immunofluorescence. One could think that the choice of another DNA stain e.g., DAPI (4,6-diamidino-2-phenylindol), might solve the problem. This may be possible in a two-light-source flow cytometer but is difficult in a one-light-source instrument because DAPI is excited between 300 and 400 nm where the fluorescein fluorescence is not optimally excitable. The use of ethidiumbromide or propidium iodide as DNA stain offers no advantage because of spectral overlap with fluorescein and the need for enzymatic pretreatment of the cells. Enzymatic treatment may, however, affect the antigenic determinants on the cell membrane.

As multiparameter flow-cytometric measurements become more sensitive they are of general interest for the identification of small numbers of tumor cells in tissue biopsies. It is thought that the simultaneous cell volume and DNA measurement is of great importance for multiparameter measurements because of the identification of morphologically intact cells, the determination of the cell cycle phase, and the distinction of inflammatory cells from parenchymal or tumor cells by these two parameters. The nonparallel expression of different antigens on the cell surface, such as CEA and EMA on cervical cancer cells, should lead to the use of antibody cocktails for the flow-cytometric identification of abnormal cells. This would further increase the sensitivity of the method. The results of such an experiment with CEA and EMA antigen are calculated in the last column of Fig. 2 from the data of the previous columns. The calculation shows the validity of this concept. The false negative rate is zero and the false positive rate 6.3% for the total of 32 samples if a threshold of more than 0.8% antigen positive cells is set as indicative for a malignant sample. The differences of CEA and EMA expression on cells of malignant samples may be of prognostic significance for the patient. It is important to increase the number of samples in future studies. Especially patients with Pap. II, III, and IV smears have to be included to test the discriminative power of this method in more difficult cases. The overlap between normal and malignant cell samples might be reduced further by exactly titering the antibodies to reduce the unspecific background of the measurement or using monoclonal antibodies. It is also possible to measure an additional cytoplasmic fourth parameter. This could be an enzyme activity (Watson 1980; Malin-Berdel and Valet 1980; Dolbeare and Smith 1979) or a hormone receptor (Daxenbichler et al. 1980; Nenci et al. 1980). The three fluorescent stains of such a measurement could be excited with a double-light-source flow cytometer. Considering the high increase of sensitivity for the detection of abnormal cells if three parameters instead of two parameters are used it is reasonable to assume that on proper choice of the fourth parameter again a significant increase of sensitivity would be achieved. The present results and the prospective possibilities of flow cytometry suggest that multiparametric measurements will be of great practical importance for clinical diagnosis and therapy control in cancer patients.

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