Cytotoxic Drug Testing in Head and Neck Cancer by Multiparametric Flow Cytometry

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• **Individual chemosensitivity assessment of solid tumors has been hindered by the lack of a reliable and rapid test system for predicting inhibition of tumor cell growth in vitro of all malignant cells and for distinguishing between inflammatory and tumor cells. Six long-term cultured and recloned squamous cell carcinoma lines of the head and neck were analyzed using a newly developed multiparametric flow cytometric technique. After incubation with cytotoxic drugs at different time intervals, each cell line showed its own chemosensitive profile. With this multiparametric staining technique, it is possible to monitor the in vitro response of tumor cells and to compare their quantitative behavior with that of nonmalignant inflammatory cells in solid tumors of the head and neck.**

(Arch Otolaryngol Head Neck Surg **1986;112:1078-1084)**

Inoperable and recurrent squamous cell carcinomas are the primary sites subjected to chemotherapy in the head and neck. According to the individual physician's experiences, either established or newly developed protocols of chemotoxic drugs are applied, without modification, to all patients who are not treatable with surgery or radiotherapy.

In vitro scoring of chemotherapeutic agents prior to therapy has been analyzed by the reduction of tritiated thymidine uptake.[1] This test has been used infrequently because both tumor cells and surrounding nonmalignant cells show thymidine uptake, masking the true tumor response. Investigation with autoradiography, which takes two to three weeks, is too time-consuming for the application of individual clinical treatment control.[2] Initially, a more predictable method seemed to be the colony formation in soft agar.[3,4] However, it soon became apparent that only a small number of tumors could be cultured[5] and that only a very small fraction of the whole tumor grew in vitro (low cloning efficiency). Thus, individual chemosensitivity assessment of solid tumors has been hindered by the lack of a reliable and rapid test system that can be used to predict inhibition of tumor cell growth of a representative cell population. Furthermore, the optimal killing dosage needed for malignant cells has to be separated from the in vitro response of nonmalignant and inflammatory cells.

For the exact identification of tumor cells with the newly developed multiparametric flow cytometric technique, the behavior of malignant cells has to be characterized. In the present study, we report our results on six recloned permanent cell lines of squamous cell carcinomas of the head and neck. The cells were stained for pH, esterase activity, and DNA content. Simultaneously, the cell volume was determined using the presence of monosized particles as an internal standard. Using cell lines as additional biologic reference, it is possible to analyze the tumor response in correlation with the response of inflammatory cells, thus offering the hope of significant predictive guidance for individual chemosensitivity testing in cancer therapy.

MATERIALS AND METHODS

Six long-term cultured squamous cell carcinomas of the head and neck were used. The following cell lines were recloned in our laboratory and cultured for more than three years: HLaC-78, HLaC-79 (larynx), and HPaC-79 (parotid gland)[6,7]; SCC-15 and SCC-25 (tongue)[8]; and FaDu (hypopharynx).[9] Since these lines were well characterized regarding their similarities to the original tumor, we used these cells after intensive recloning to obtain a homogeneous population. The cells were cultured in basal medium Eagle (BME) or minimal essential medium (MEM, Dulbecco, Boehringer, Mannheim, West Germany) containing 5% fetal calf serum (Gibco Europe, Karlsruhe, West Germany) supplemented with 1% penicillin G sodium and streptomycin sulfate ([100 000 U/mg/mL] Seromed, West Berlin), 1% of 200 mmol L-glutamine (×100), and 1% of 1 mol HEPES buffer at a pH of 7.3 (Gibco). The cells were kept in a humidified incubator with 5% carbon dioxide at 37°C. When

Accepted for publication March 21, 1986.

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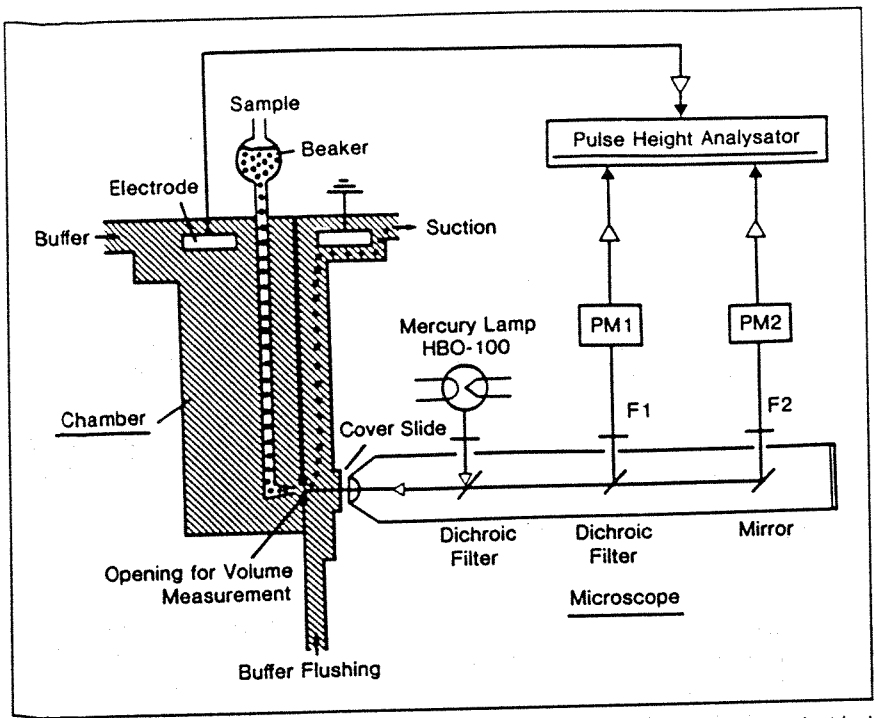

Fig 1.—Schematic graph of flow cytometer (Fluvo-Metricell) suitable for simultaneous electrical measurement of cell volume and optical determination of two cell fluorescences of different wave length. F1 indicates filter short pass, 440 nm, and long pass, 418 nm; F2, filter long pass, 500 nm; PM1, photomultiplier, 418 to 440 nm; and PM2, photomultiplier, 500 to 700 nm.

confluent monolayers at a density of 5×10^5 cells per milliliter were reached, the adherent cells were detached with 0.05% trypsin and 0.02% EDTA (Boehringer, Mannheim, West Germany) for 30 s to five minutes, depending on the adherence of each line. The cells were washed twice in culture medium, and then 20 μL of each cytotoxic drug was added to 2 mL of the cell suspension containing 2×10^5 cells. The cell suspensions were incubated in microtiter plates for a period of four days. After in vitro culture, the cells were quantitatively removed by scraping and vigorous rinsing with TRIS-buffered hydrochloride (TBS). Then, 200 μL of each suspension was cytocentrifuged and stained with Papanicolaou's stain for morphological analysis.

Aliquots (250 μL) of the cell suspensions were stained for pH and esterase activity with 5 μL of the esterase substrate 1,4-diacetoxy-2,3-dicyanobenzene (ADB, Paesel, Frankfurt, West Germany) and dissolved at a concentration of 1 mg/mL in dimethylformamide to obtain a final concentration of 20 μg/mL ADB in the cell suspensions. The nuclei of the dead cells were simultaneously counterstained with 5 μL of 2 mg/mL propidium iodide (Serva, Frankfurt, West Germany) solution in 0.15 mol sodium chloride buffered with 10 mmol TBS to a pH of 7.35. The cells were incubated for five to ten minutes at 4°C.

Porous, amino-group-bearing monosized latex particles (2.5×10^5 particles per milliliter), with a diameter of 4.88 μm,[10] were prestained with 20 μg/mL of 1,4-dicyano-2,3-hydrochinon (DCH, EGA-Chemie, Steinheim, West Germany) and added as internal concentration, fluorescence, and size standard. Simultaneously, the cell volume, the blue/green esterase (pH) and the red (DNA) fluorescence of the double-labeled cells were determined in a flow cytometer (Fluvo-Metricell) (Fig 1). The volume of each cell was measured electrically at a current of 0.229 mA in a TBS-filled chamber. The sample beam was focused hydrodynamically through the center of a cylindrical orifice that was 100 μm in length and 95 μm in diameter. Thus, the fluorescence and the volume were measured at a speed of 500 to 1500 cells per second. From a high-pressure mercury arc lamp (HBO-100), the dye fluorescence was excited between 300 and 400 nm just beyond the orifice. The blue fluorescence was collected between 418 to 440 nm and the green and red fluorescence between 500 and 700 nm with two photomultiplier tubes using dichroic filters (Zeiss, Oberkochem, West Germany) to separate both fluorescence ranges. The electrical cell volume and fluorescence signals were amplified by 2.5-decade logarithmic amplifiers. The data were stored in list mode on magnetic tape and subsequently analyzed by computer programs (FORTRAN).[11]

RESULTS

The tumor cells of the squamous cell carcinoma line SCC-25 and the monosized calibration particles can be seen in the three-dimensional cube representation of the simultaneous cell volume and double-fluorescence measurement (Fig 2, left). The tumor cells are shown as a cloud of large cells with high-esterase activity. Dead cells, however, manifest low-esterase activity and stainability with the DNA dye propidium iodide, which makes a clear separation possible. The particles represent a distinct cloud with a small volume and low blue and green fluorescence. Fluorescence of esterase activity and DNA content are indicated in arbitrary units. The diagram is standardized to the maximum channel content, which was 236 particles per histogram channel. The contour lines of the maximal channel content are plotted around the channels that contain three and one particles. By this simultaneous display, the information of the position of the vast majority of all cells is obtained for quantitative evaluation.

By comparing the control assay shown in Fig 2, left, with the aclarubicin-treated (16 μg/mL, four days at 37°C) assay shown in Fig 2, right, the cytotoxic drug effect can be quantitatively seen. Almost all vital squamous carcinoma cells of line SCC-25 have disappeared. As a consequence of cytotoxicity, the number of dead cells increased significantly. Similar histograms were obtained with the remaining five cell lines and other chemotherapeutic agents (data not shown). The drug concentrations in the assay were ten times the concentrations of the drugs measured in blood plasma of patients during clinical therapy.

The projection of the cube data (Fig 2) of vital cells and the calibration particles onto a cell volume against esterase activity plane allows the distinction of three compartments (Fig 3). The reference particles peaked in the small-volume compartment with low fluorescence. The tumor cells (SCC-25), however, appeared almost

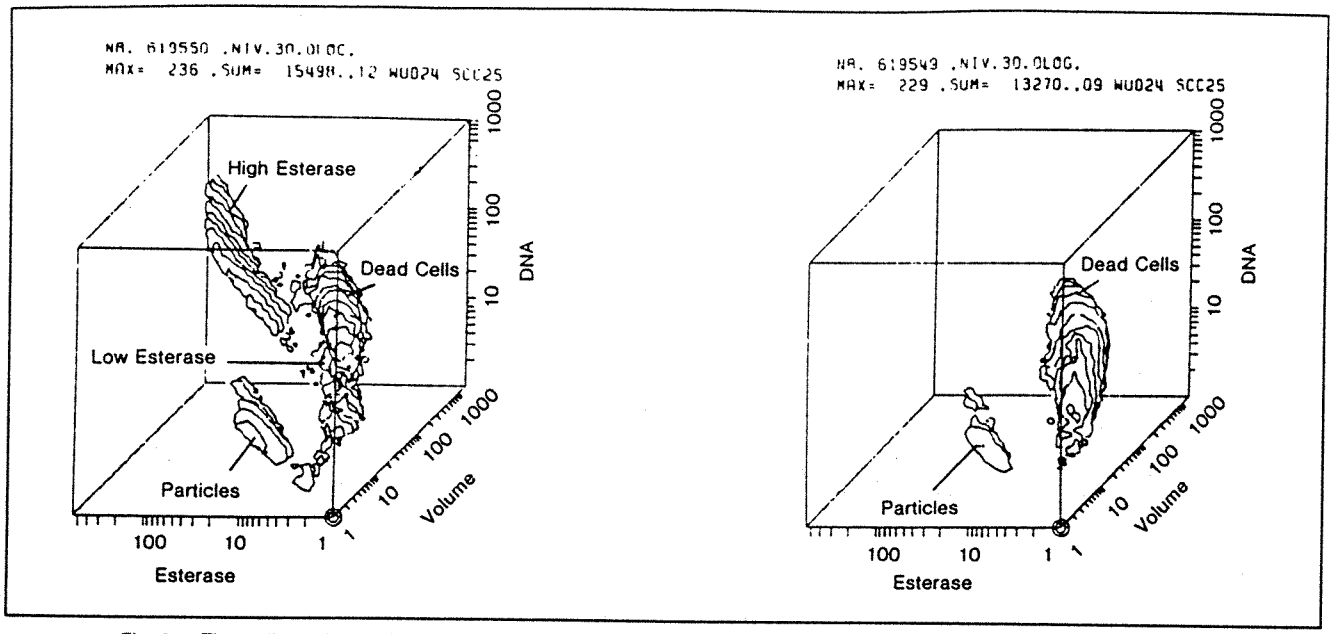

Fig 2.—Three-dimensional distribution of squamous cell carcinoma line SCC-25. Left, Total of 15 498 cells was measured. Right, After four days of incubation with aclarubicin (16 μg/mL), virtually all high-esterase-staining cells disappeared, and peak of dead cells increased. Total of 13 270 cells was measured.

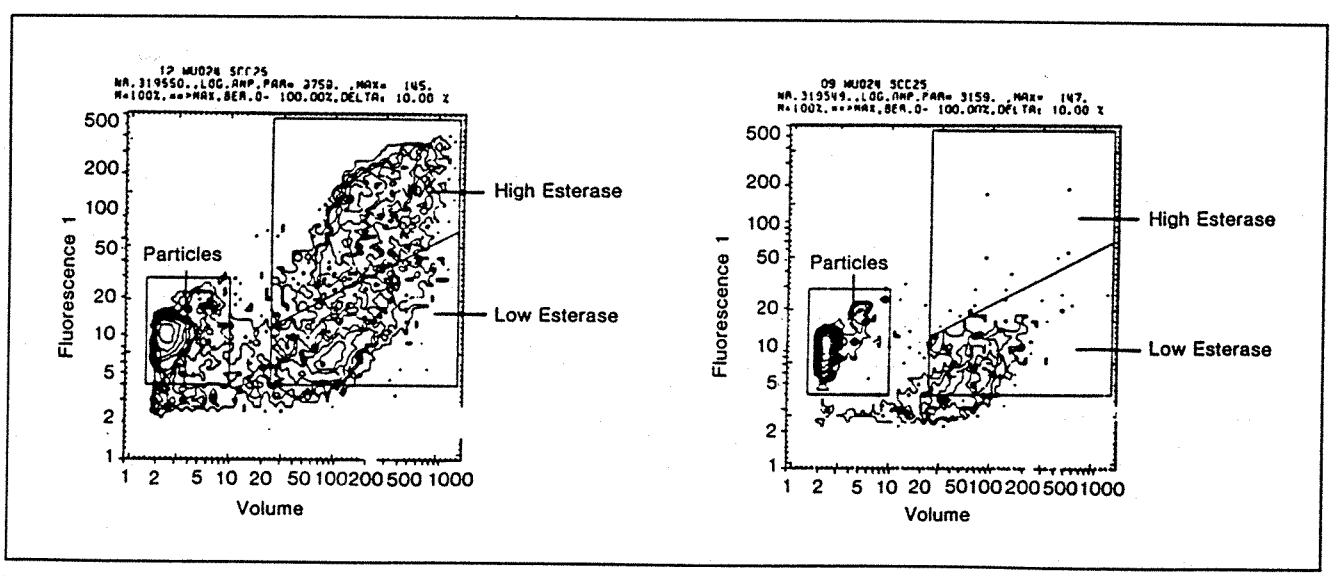

Fig 3.—Cell volume vs esterase activity plot of squamous cell carcinoma line SCC-25. High- and low-esterase activities are distinguishable in large-volume compartment. Total of 9759 cells was measured in control (left), compared with 3159 cells after four-day incubation with aclarubicin (right).

exclusively in the large-cell compartment with high-esterase activity. After incubation with aclarubicin, the tumor cells disappeared almost completely. The logarithm of each histogram was divided into ten equal steps, and a contour line was plotted for each step. The lowest contour lines represent channels containing one cell, the highest contour lines represent channels of 145 cells per histogram channel. Most of the tumor cells with high-esterase activity disappeared after incubation with aclarubicin (16 μg/mL) for four days (Fig 3, right). Monosized particles were used as an internal standard. For quantitative evaluation, the cell and particle contents of the various compartments were calculated. The fluorescence scales are given in relative fluorescence units. One unit of the volume scale corresponds to 25 μm^3. Corresponding results were obtained with other cytotoxic drugs and cell lines (data not shown).

Bearing in mind the logarithmic scale, the tumor cells showed large volumes. In the large-volume compartment with low-esterase activity,

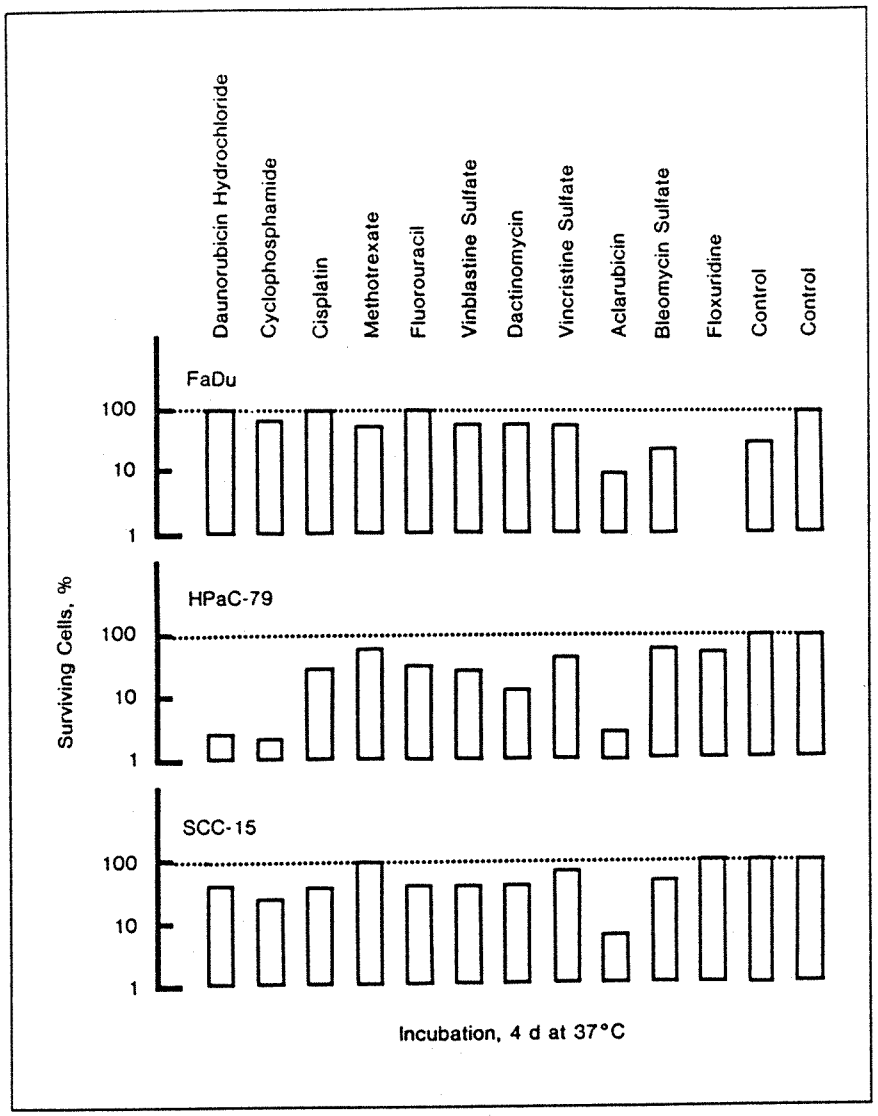

Fig 4.—Chemosensitive response of in vitro cultured squamous cell carcinoma lines (FaDu, HPaC-79, and SCC-15) of head and neck. Cells were incubated with various drugs for four days, and individual reaction profiles of each cell line are shown. Concentrations are as follows: daunorubicin hydrochloride, 10 μg/mL; 4-hydroxy-cyclophosphamide, 140 μg/mL; cisplatin, 10 μg/mL; methotrexate, 10 μg/mL; fluorouracil, 150 μg/mL; vinblastine sulfate, 1 μg/mL; dactinomycin, 1 μg/mL; vincristine sulfate, 100 ng/mL; aclarubicin, 16 μg/mL; bleomycin sulfate, 3 μg/mL; and floxuridine, 3 μg/mL.

cells are found that are in the process of dying but that are not yet permeable for the DNA dye propidium iodide. Dead cells can be quantitatively separated from the vital cells by computer analysis (Fig 2). The compartment between the small- and large-volume compartment was nearly empty. It comprises the inflammatory cells, mainly lymphocytes and granulocytes, in the clinical samples that are described in human ovarian and cervix uteri cancers.[30] In preliminary experiments with tumor biopsy specimens from cancer patients with squamous cell carcinomas in the head and neck, this compartment was indeed filled with inflammatory cells. Thus, the different functional reactions of individual cell populations will be measurable in future experiments with clinical tumor explants. It should be emphasized that, with the exception of mechanical disruption, the humoral and cellular microenvironment of the tumor cells can be largely imitated in vitro during the functional analysis, since the patient's own serum is used for the cell culture. In addition, a low oxygen condition (2% to 5%) in the presence of 5% carbon dioxide (CO_2) and 90% to 93% nitrogen (N_2) may be created.

In Fig 4, the quantitative evaluation of the cytotoxic drug experiments with the squamous cell carcinoma lines FaDu, SCC-15, and HPaC-79 is described. The control cultures are assayed before and after the four-day incubation period at 37°C. All recloned cell lines reacted strongly with aclarubicin. The cell line FaDu showed an additional response to bleomycin sulfate, while SCC-15 showed a response to 4-hydroxy-cyclophosphamide. Strikingly, the cell lines did not show the same chemosensitivity for the most commonly used drugs in chemical protocols, such as cisplatin, fluorouracil, methotrexate, or bleomycin sulfate. Thus, the use of human permanent cell lines for screening of new chemotherapeutic-active drugs or to establish new clinical therapy protocols may be questionable.

Additional information is gained from the simultaneous three-parameter measurement shown in Fig 1, left, by calculation of one-parameter distributions. Thus, representative one-parameter histograms of the squamous cell carcinoma line SCC-25 derived from a tongue cancer are displayed in Fig 5. Figure 5, top left, shows the distribution of the DNA content of dead cells stained with propidium iodide. The data are plotted as percentage of the maximum frequency in the histogram channels, which was 783 particles. The first peak represents the cells in the G_0/G_1 phase of the cell cycle, while the second peak contains cells in the G_2M phase. Between both peaks, cells in the S-phase can be identified. The G_0/G_1- and G_2M-phase cells are lying close together due to the logarithmic amplifiers used for the fluorescence measurements. To the left side of the G_0/G_1 peak, cells with degraded DNA are visible.

Although the cell cultures are started from a single-cell suspension, the cells develop desmosomes between their cell membranes.[29] After detachment of the monolayer, the adherent

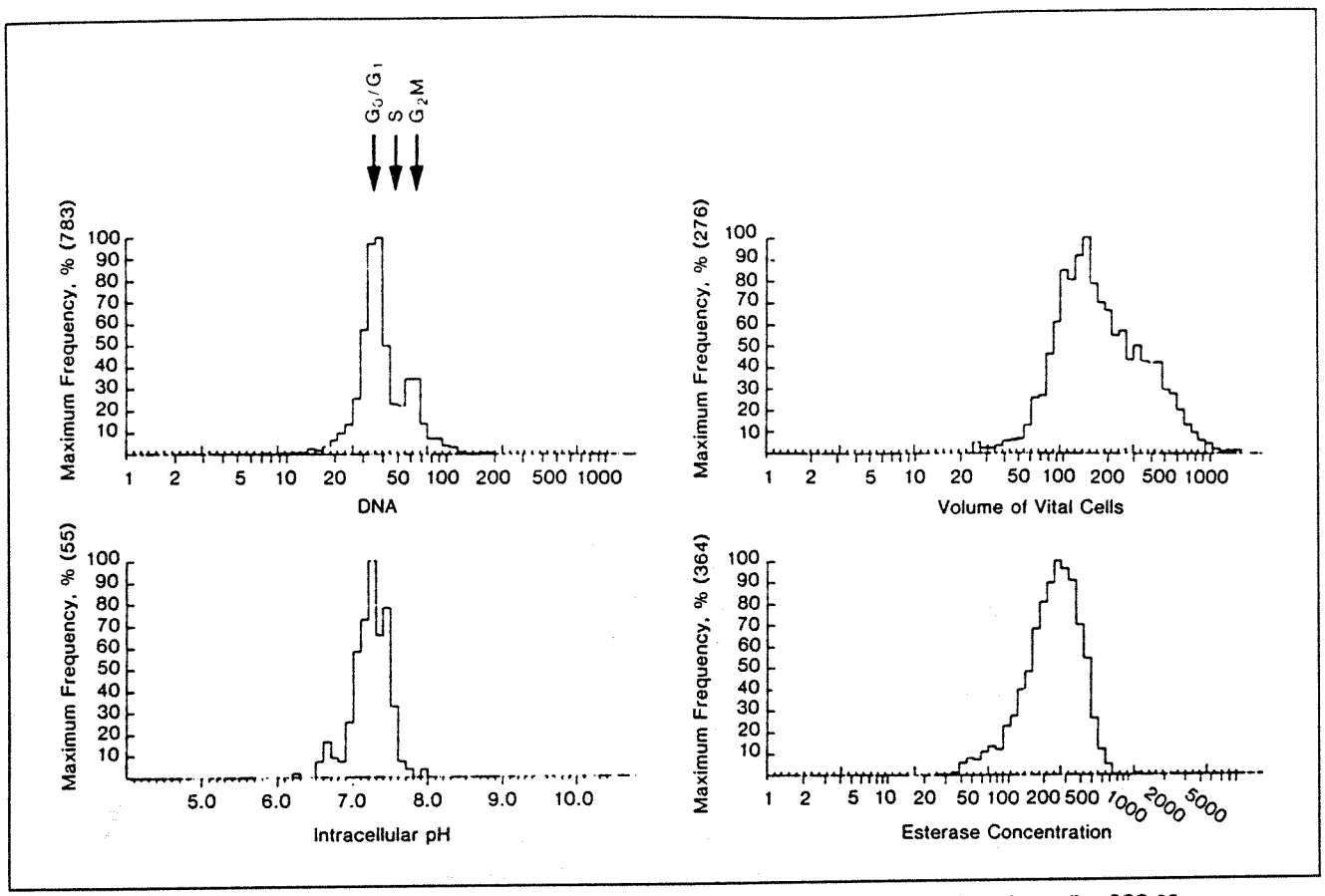

Fig 5.—Cell-volume distribution, esterase activity, pH, and DNA content of squamous cell carcinoma line SCC-25.

cells stick close together, forming aggregates. By repipetting with Pasteur pipettes, the cell aggregates are disrupted. Cell aggregates are not a major problem, since no peak at the triploid DNA content, which is the usual indicator of cell aggregates, was seen. In preliminary experiments with patient samples of solid squamous cell carcinomas of the head and neck, only a few aggregates were obtained after accurate mechanical preparation of single-cell suspensions.

In Fig 5, top right, the cell volume distribution of the squamous cell carcinoma line SCC-25 is displayed. The data are standardized as percentage of the maximum channel frequency of 276 particles per histogram channel. The cell-size profile of these vital cells varies about one decade. This reflects the heterogeneity of the tumor cells, despite an intensive recloning. The large-cell volume enables the distinction of the tumor cells in patient samples from inflammatory cells of the hematopoietic system (lymphocytes and granulocytes), which have a modal volume of 280 μm^3, compared with the tumor cells of approximately 3000 μm^3.

Figure 5, bottom left and bottom right, represent the intracellular pH and esterase-activity distribution of the vital cells. The intracellular pH of the tumor cells varies from 6.9 to 7.6 and is calculated from the blue and green fluorescence ratio. The esterase activity in arbitrary units was calculated as the ratio of the blue fluorescence of the vital cells divided by their cell volume. From these data, it is possible to monitor the intracellular biochemical alterations of the vital cells during chemotherapeutic drug testing in vitro.

COMMENT

Analysis of cell-cycle phase distribution patterns with tritiated thymidine incorporation into DNA have not been successful because of the complexity of the tumor tissue,[12] which, besides malignant cells, also contains connective tissue and diploid inflammatory vascular cells. This leads, however, to difficulties in interpreting the altered cell-cycle distribution caused by cytotoxic drugs.

In a different approach, tumor biopsy specimens have been cultured in a soft agar, allowing the formation of tumor cell colonies. Tumor cells were grown in Petri dishes,[3,4] tubes,[13] or capillaries[5,14] in the presence of cytotoxic drugs. Difficulties with prediction of sensitivity or resistance arose because only a few tumors and only a small fraction of tumor cells within the tumors formed colonies.[5]

As an alternative model for chemotherapeutic studies, the nude mouse system has not gained widespread popularity, because only 20% to 30% of the transplanted squamous cell carcinomas formed established lines.[15-22] Furthermore, heterotransplantation resulted in selection of aneuploid cell clones,[18-20] which favor the growth of biologically aggressive tumors.[21,22] Concomitantly, with the clonal com-

position, differences between the heterotransplanted and the original human tumors have been shown with respect to their growth kinetics.[16]

Two-parameter flow cytometry has only been used for determination of DNA content in head and neck cancer. Wannenmacher et al[23] demonstrated an increase of G_1M cells after ten hours and a decrease of G_2M cells 14 hours after infusion with fluorouracil. Schumann et al,[24] using ethidium bromide as a stain, described a partial arrest of human squamous epithelial carcinoma cells in G_2M (an increase from 3% to 11%) 30 hours after two doses of bleomycin sulfate (15 ng per dose). Similarly, Ganzer et al[25] showed alterations of the cell cycle after an attempt with partial synchronization of tumor cells with fluorouracil. Lampe et al,[26] used a propidium iodide stain for the nuclear ploidy in 32 patients with a squamous cell carcinoma of the upper aerodigestive tract, indicating a lower prognosis when aneuploidy was present. Only recently, Ensley et al[27] determined the optimal preparative and staining technique in head and neck cancer with propidium iodide. Kaiser et al[28] measured the heterogeneity of four squamous cell carcinoma lines after incubation with various chemotherapeutic drugs, but without control of the heterogeneous growth characteristics that occur during in vitro culture of recloned cell lines of the head and neck.[29]

The advantage of the present method is the close similarity of the biologic condition to the patient environment with regard to the cellular and humoral composition of the samples, as patient specimens are optimally cultured in the presence of autologous serum (data not shown). In addition, multiparametric flow cytometric analysis allows distinction between small inflammatory cells, which are predominantly lymphocytes, and large cells, which are almost exclusively tumor cells.[30] The analytical separation between tumor cells and epithelial or mesenchymal cells is possible since tumor cells are mostly aneuploid by measurement of DNA content. If there is no aneuploidy of the tumor cells, no direct identification of tumor cells is possible within the large-cell compartment. Concomitantly, morphological examination of cytocentrifuged preparations allows the determination of the percentage of tumor cells present.

Besides the quantitative evaluation of surviving and dead cells under therapy, the method offers, with no additional measurements, a multitude of biochemical and physiological data on DNA distribution (aneuploidy), esterase activity, intracellular pH, and cell volume. Dead cells are identified by their ability to stain with propidium iodide. Vital cells stain with the esterase and pH dye ABD. In preliminary experiments with patients who have squamous cell carcinoma of the head and neck, during primary culture with or without cytotoxic drugs, no overgrowth of fibroblasts could be observed; predominantly, tumor cells and lymphocytes survived the culture conditions, which is favorable for clinical use of this assay.

With the technique described, chemosensitive responses in vitro to an individual agent, a drug combination, sequential drug combinations, or radiation may be tested. Sensitivity to an in vitro treatment is judged by a 50% reduction of the large-cell compartment and is compared with the control values. In addition, drug resistance can be predicted. As the stain does not need any centrifuge steps, the method is suitable for complete automation, especially since there are newly developed microprocessor instruments for readjustments during the repeated measurements and since controlled shutdown in case of orifice plugging is possible, to avoid sample loss.

The addition of monosized latex particles in the dye serves both as an internal concentration standard and for the calibration of the cell volume and fluorescence scales. This has only been possible since monosized particles with diameters greater than 2 μm have become available.[10] The rapid automated analysis opens new possibilities in monitoring the functional behavior of the cells and in altering individual clinical protocols during therapy. In the future, multiparametric flow cytometry will enable the determination of cellular proteins, intracellular enzyme activities, lipids, cell surface antigens, and receptors. Thus, pathological conditions and metabolic changes can be detected earlier by analyzing the biologic state of each cell compartment in head and neck tumors.

Multiparametric flow cytometry is of great importance because morphologically intact cells can be identified, the cell-cycle phase can be determined, and inflammatory cells can be distinguished from parenchymal and tumor cells. In addition, only small sample sizes are needed because all tumor cells are analyzed. By using several parameters simultaneously, this method will provide better understanding of the biochemical and immunologic characteristics and of the cellular interactions within the heterogeneous population in solid tumors. Furthermore, multiparametric flow cytometry will offer increasing knowledge and potential for clinical diagnosis and control during chemotherapy and radiotherapy in patients with head and neck cancer.

The cell lines used in our study were kindly provided by H. P. Zenner, MD, PhD, Würzburg, West Germany (HLaC-78, HLaC-79, and HPaC-79); J. Rheinwald, PhD, Boston (SCC-15 and SCC-25); and J. Fogh, PhD, New York (FaDu); and the amino-group-bearing monosized latex particles, by J. Ugelstad, Trondheim, Norway.

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