Multiparametric flow cytometry of human squamous cell carcinoma lines from the head and neck

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Squamous cell carcinomas of the head and neck consist of heterogeneous cell populations. The purpose of the present study was to investigate whether established cell lines from human head and neck cancers under chemotherapy behaved similarly to tumors in patients during in vivo treatment. This is of interest in terms of improvements of chemotherapeutic protocols and understanding of the mechanisms of cytotoxic drug resistance. Permanent squamous carcinoma cell lines of the larynx (HLaC 78, 79), parotid gland (HPaC 79), tongue (SCC-15, SCC-25), hypopharynx (FaDu), and tumor lines with different histology and origin, as mucoepidermoid cancer cells of the submandibular gland (A 253), Epstein-Barr virus-infected human B cells (BC-1) and mouse fibroblasts (3T3) were incubated with chemotherapeutic drugs for 1 to 4 days at 37° C. Despite the microscopic similarities to patient carcinomas, cancer cell lines of the head and neck showed different susceptibilities to cell kill mediated by chemotherapeutic drugs, as compared to in vivo therapeutic results with patients. The nonsquamous carcinoma lines demonstrated high chemosensitive responses after incubation with daunorubicin, cyclophosphamide, dactinomycin, vincristine, and aclarubicin. Surprisingly, only low cell killing rates in squamous carcinoma cell lines were observed after incubation with chemotherapeutic agents such as *cis*-platinum, 5-fluorouracil, methotrexate, or bleomycin, which are most commonly used for head and neck cancers. The results show that cytotoxic drug action on in vitro cultured squamous carcinoma cell lines of the head and neck is not representative for the in vivo responses of patient tumors. The cell lines are, however, of potential value for evaluation of cell biochemical changes associated with cytotoxic drug resistance. (OTOLARYNGOL HEAD NECK SURG 1988;98:552.)

Patients with squamous cell carcinoma of the head and neck, with advanced local tumor extension and massive regional nodal disease, are primary candidates for chemotherapy. In addition, patients with unresectable recurrences, with high internal risk factors for adequate operations, or with refusal for surgical removal of the tumor are considered for cytotoxic treatment in combination with radiotherapy. At present, chemotherapy is mostly given in combined sequential courses; most effective are *cis*-platinum and 5-fluorouracil, as shown by many clinical trials. Only recently, efficient simultaneous radio-chemotherapeutic programs for advanced head and neck cancer, with drugs including *cis*-platinum and 5-fluorouracil, have appeared.[1-3] In most instances, established chemotherapeutic protocols are used for all patients, with no individual modifications. Since some head and neck cancers shrink tremendously—whereas others do not respond at all to chemotherapy—it is of great interest to determine the individual chemotherapeutic sensitivity and resistance of cancer cells before the onset of treatment.

We have recently used multiparametric flow cytometry to analyze the effects of chemotherapeutic drugs on cells of patients with squamous cell carcinomas of the head and neck.[4] In this report, we were interested in whether squamous carcinoma cell lines of the head and neck and cell lines with other histologic diagnoses and other origins reacted in vitro in a manner similar

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Table 1. Concentrations of cytotoxic drugs for the in vitro assays

Drug concentrations		
1	Daunorubicin hydrochloride	10 μg/ml
2	4-hydroxy-cyclophosphamide	140 μg/ml
3	*Cis*-platinum	10 μg/ml
4	Methotrexate	10 μg/ml
5	5-fluorouracil	150 μg/ml
6	Vinblastine sulfate	1 μg/ml
7	Dactinomycin	1 μg/ml
8	Vincristine sulfate	100 ng/ml
9	Aclarubicin	16 μg/ml
10	Bleomycin sulfate	3 μg/ml
11	Floxuridine	3 μg/ml
12	Medium control	
13	Medium control	

to cytotoxic drugs as compared to known responses in patients. This would be useful to optimize therapy protocols, to screen in vitro new drugs, and to investigate the mechanism of cytotoxic drug resistance.

Our experiments demonstrate that in vitro cultured squamous carcinoma cell lines do not show the same sensitivity and resistance pattern as squamous cell carcinomas in patients.

METHODS AND MATERIALS

Recloned squamous cell carcinoma lines of the larynx (HLaC 78, HLaC 79), of the parotid gland (HPaC 79),[5] of the tongue (SCC-15, SCC-25),[6] and of the hypopharynx (FaDu)[7] were used. The head and neck cell lines were compared with those of other histologic and local origins, such as 3T3 (mouse fibroblasts), A 253 (mucoepidermoid cancer of the submandibular gland), and BC-1 (Epstein-Barr virus-infected B cells).[8] The cells were kept in 25-ml tissue-culture flasks with BME Earle, MEM Dulbecco (1x) or RPMI-1640 medium (Boehringer, Mannheim, Germany) supplemented with 5% fetal calf serum (Gibco Europe, Karlsruhe, Germany), 1% penicillin/streptomycin (10,000 E/mg/ml; Servamed, Berlin, Germany) and with 1% 200 mM L-glutamine (Gibco). To 2 ml cell suspension with 2×10^5 cells, 20 μl of each cytotoxic agent were added. The drug concentrations were adjusted to ten times the plasma concentrations during clinical use for all assays (Table 1). The adherent growing squamous carcinoma cell lines, including the mucoepidermoid cancer line A 253, were detached with 0.5 ml trypsin/EDTA (0.05/0.02%; Boehringer, Mannheim, Germany). Cell number and viability was determined microscopically in Neubauer counting chambers. The cells were analyzed in the Fluvo-Metricell,[4,9,10] before and after a 4-day incubation period, in air with 5% CO_2 at

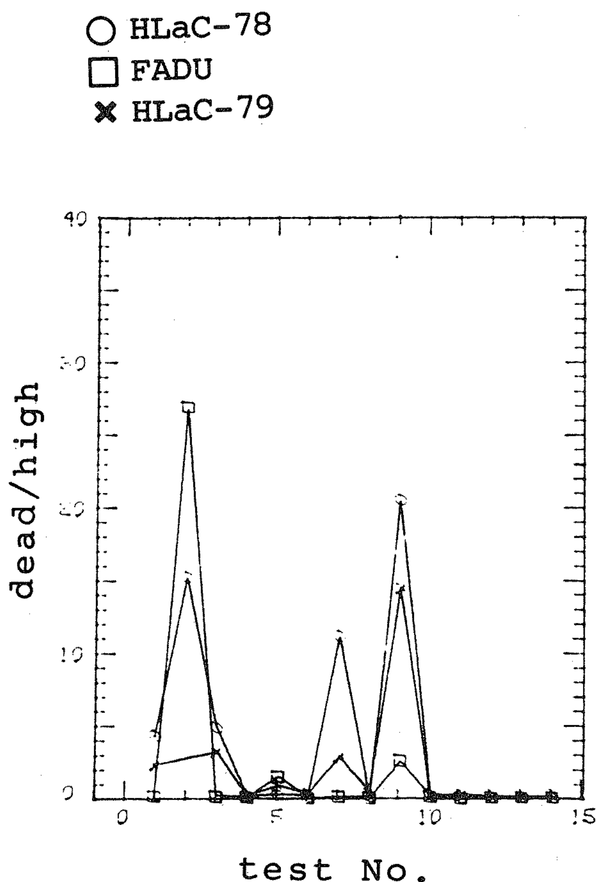

Fig. 1. Ratio between dead cells and highly esterase positive, vital cells from squamous cell carcinoma lines HLaC 78 (n = 4) and HLaC 79 (n = 4) of the larynx and FaDu (n = 2) of the hypopharynx after incubation with cytotoxic agents in percent of control assays without cytotoxic drugs. The standard errors of the ratios were between ±8% and 32% of the values.

37° C. 50 μl aliquots of all samples were cytocentrifuged and stained according to Papanicolaou.

For the flow cytometric experiments, 250 μl of each cell suspension were incubated with 20 μg/ml ADB (1.4-diacetoxy-2.3-dicyanobenzene; Paesel, Frankfurt, Germany) of the intracellular esterase and pH indicator dye for 10 minutes at room temperature.[11] The DNA of dead cells was stained simultaneously with propidium iodide (40 μg/ml; Serva; Frankfurt, Germany). Porous, monosized particles with diameters of 4.88 μm[12] were added to each sample as internal standard. The particles were prestained with 20 μg/ml DCH (1.4-dicyano-2.3-hydrochinon; Ega-Chemie, Steinheim, Germany). They served as internal counting standard and as reference for volume and fluorescence intensities. The following number of experiments with all the drugs given in Table 1 were performed with different cultures on different days:

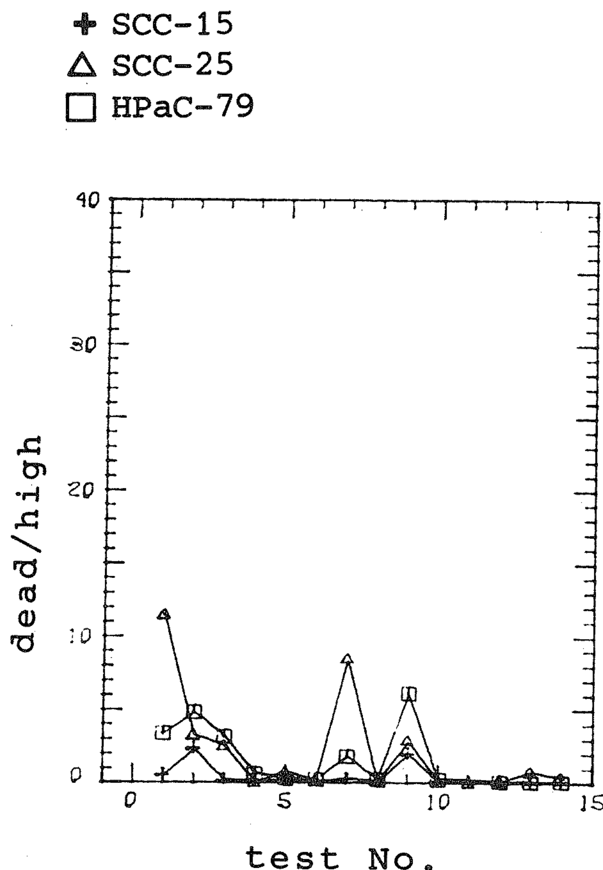

Fig. 2. Ratio between dead cells and highly esterase positive, vital cells with squamous cell carcinoma lines SCC-15 (n = 5) and SCC-25 (n = 5) of the tongue and HPaC 79 (n = 5) of the parotid gland after incubation with cytotoxic agents in percent of control assays without cytotoxic drugs. The standard errors of the ratios were between ±14% and 26% of the values.

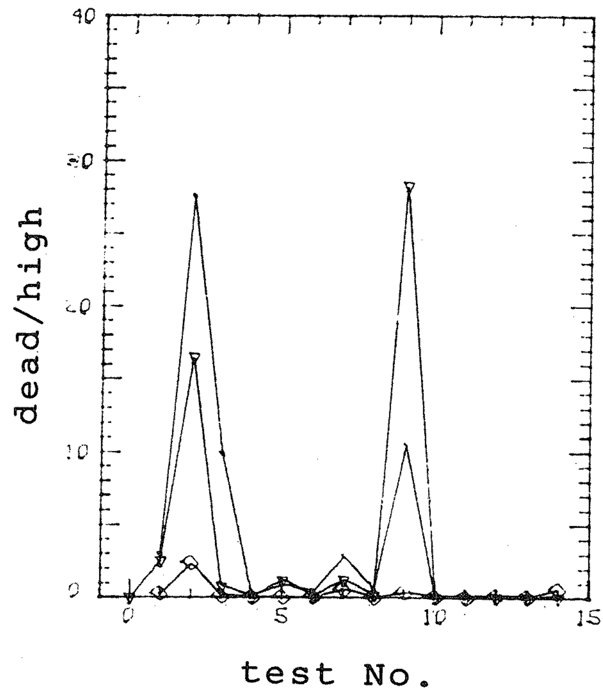

Fig. 3. Ratio between dead cells and highly esterase positive, vital cells from the mucoepidermoid cancer line (A253; n = 5) of the submandibular gland, of the Epstein-Barr virus transformed human B cell line (BC-1; n = 2) and of the mouse fibroblast line (3T3; n = 3) after incubation with cytotoxic agents in percent of control assays without cytotoxic drugs. The standard errors of the ratios were between ±15% and 35% of the values.

HLaC 78, 79 (n = 4); SCC-15, SCC-25 (n = 5); HPaC (n = 5); FaDu (n = 2); A 253 (n = 5); BC-1 (n = 2); and 3T3 (n = 3). From each drug concentration and cell line a minimal number of 5000 cells was measured in the flow cytometer performed. All data were stored on list mode on magnetic tapes for subsequent analysis by Fortran computer programs.[13] For better graphic demonstration, the mean values of each cell line were plotted in a non-mathematic fashion by interconnecting the results of the different cytotoxic drugs. The data were evaluated in the following way: ratios between dead and vital cells were calculated as a measure of killing efficiency for each cytotoxic drug and for the controls. The ratios of the cytotoxic drug-treated assays were then expressed in percent of the ratios of the control assays and plotted as mean values (Figs. 1 through 6).

RESULTS

The experiments were divided into three groups according to tumor histology and origin of the cell lines: *first,* squamous carcinoma cell lines of the larynx (HLaC 78 and 79) and hypopharynx (FaDu); *second,* squamous carcinoma cell lines of the oral cavity (SCC-15 and SCC-25) and parotid gland (HPaC 79); and *third,* cell lines from other origins (Epstein-Barr virus-infected B cells = BC-1, mouse fibroblasts = 3T3) or with other histologic characteristics (mucoepidermoid cancer of the submandibular gland A 253).

The chemosensitive response of all cell lines showed an increase in the ratio between dead cells and highly esterase positive, vital cells only after incubation with cyclophosphamide, dactinomycin, and aclarubicin. The larynx and hypopharynx lines (HLaC 78 and FaDu)

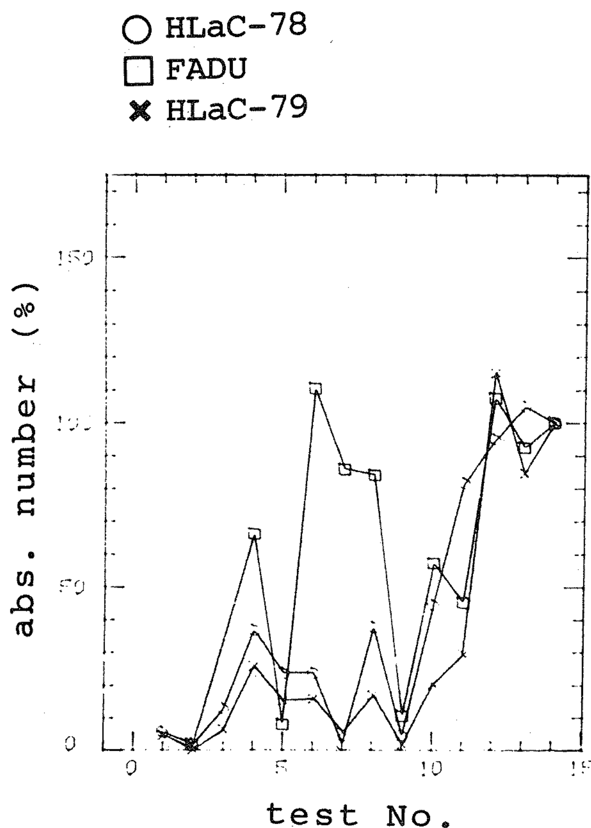

Fig. 4. Surviving cells of squamous cell carcinoma lines HLaC 78 (n = 4) and HLaC 79 (n = 4) of the larynx and FaDu (n = 2) of the hypopharynx after incubation with cytotoxic agents in percent assays without cytotoxic drugs. The standard errors of the values were between ±12% and 29%.

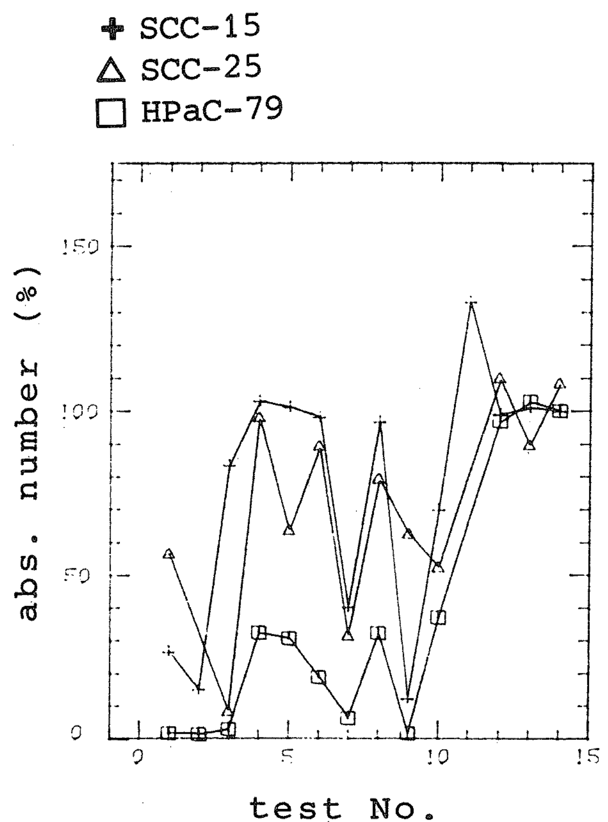

Fig. 5. Surviving cells of squamous cell carcinoma lines SCC-15 (n = 5) and SCC-25 (n = 5) of the tongue and HPaC 79 (n = 5) of the parotid gland after incubation with cytotoxic agents in percent of control assays without cytotoxic drugs. The standard errors of the values were between ±6% and 33%.

reacted strongly with cyclophosphamide; HLaC 78 reacted with dactinomycin and aclarubicin; and HLaC 79 reacted with aclarubicin (Fig. 1). The squamous carcinoma cell lines from the oral cavity and parotid gland reacted with significantly lower cell killing rates (Fig. 2). The cytotoxicity for the nonsquamous carcinoma cell lines was high with cyclophosphamide and aclarubicin in BC-1 and 3T3 cell lines, whereas the mucoepidermoid cancer line (A 253) of the submandibular gland did not react to any of the chemotherapeutic agents used in this study (Fig. 3). In a comparison of the cell lines from the larynx and hypopharynx with those of the oral cavity and parotid gland, surprisingly the cell lines from the later locations showed significantly reduced response rates, although all lines were classified microscopically as squamous cell carcinomas.

When the absolute number of surviving cells in percent of the control assays were analyzed, marked differences were again observed (Figs. 4, 5, and 6). The larynx lines HLaC 78 and 79 exhibited similar reaction profiles. The hypopharynx line (FaDu) differed after treatment with methotrexate, vinblastine, dactinomycin, and vincristine. Analysis of the oral cavity cell lines (SCC-15, SCC-25) revealed the same cytotoxicity, except for *cis*-platinum, 5-fluorouracil, aclarubicin, and floxuridine. Interestingly, the squamous carcinoma cell lines of the parotid gland (HPaC 79) resulted in a curve similar to the two lines of the larynx (HLaC 78 and 79). In studying the nonsquamous carcinoma cell lines, the mucoepidermoid cancer of the submandibular gland (A 253) exhibited a decrease to 50% of surviving cells after treatment with cyclophosphamide, dactinomycin, vincristine, and aclarubicin. From the BC-1 line, hardly any cells survived the incubation with different drugs. Surprisingly, the highest cell killing of the head and neck cancer cell lines was not observed after incubation with the most commonly used chemotherapeutic agents,

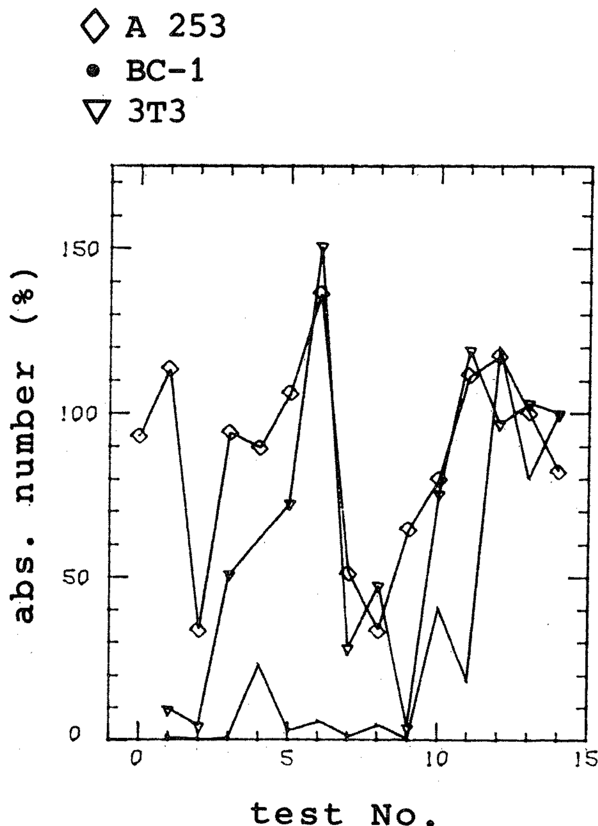

Fig. 6. Surviving cells of the mucoepidermoid cancer line A253 of the submandibular gland (n = 5), of the Epstein-Barr virus transformed human B cell line (BC-1; n = 2) and of the mouse fibroblast line (3T3; n = 3) after incubation with cytotoxic agents in percent of control assays without cytotoxic drugs. The standard errors of the values were between ±14% and 39%.

such as *cis*-platinum, 5-fluorouracil, methotrexate, or bleomycin.

Reproducibly, the squamous carcinoma cell lines of the larynx and oral cavity demonstrated a reduced cell volume between 30% and 65% of the control values after treatment with cyclophosphamide, dactinomycin, and aclarubicin. The larynx cancer line (HLaC 78) showed a decreased cell volume (63%) with 5-fluorouracil, whereas in the hypopharyngeal line (FaDu) elevated cell volumes (133%, 128%) in comparison to the controls were observed with 5-fluorouracil and vinblastine.

Esterase activities of vital cells were increased in larynx HLaC 78 cells (129% to 150%) for cyclophosphamide, vinblastine, vincristine, and dactinomycin and decreased (42% to 60%) in the hypopharynx line (FaDu) for daunorubicin, cyclophosphamide, dactinomycin, and aclarubicin.

Interestingly, a marked alkalinization of surviving cells after cytotoxic treatment was observed. The normal pH values (±SE) were pH = 6.63 ± 0.04 (FaDu); pH = 6.60 ± 0.10 (HLaC 78); pH 6.68 ± 0.12 (HLaC 79); pH = 6.64 ± 0.16 (HPaC 79); pH = 6.57 ± 0.12 (SCC-15); pH = 7.07 ± 0.15 (SCC-25); pH = 6.85 ± 0.20 (A 253); pH = 6.80 ± 0.14 (BC-1); and pH = 6.54 ± 0.19 (3T3). Alkalinization between 0.4 and 0.8 pH units were measured cytofluorometrically in the surviving HPaC 79, SCC-25, BC-1, and 3T3 cells after treatment with daunorubicin, cyclophosphamide, dactinomycin, and was most prominent after aclarubicin.

DISCUSSION

Neoplasms consist of tumor cells with marked differences in phenotype, karyotype, and clinical behavior.[14] The differences, found within one tumor, between the tumor and its metastases, and between tumors of the same histologic type in different patients, are known as tumor heterogeneity. This reflects the appearance of malignant cells at different stages of differentiation and the coexistence of different clonal cell populations.[15] The cancer cells may differ in characteristics, such as morphology, metastatic potential, immunogenicity, growth kinetics, and resistance to cytotoxic drugs. Heterogeneity seems to be the result of neoplastic progression, regardless of the uni- or multicellular origin[16]; this results in rapid generation of clonal subpopulations with altered phenotypic determinants.[17] Furthermore, new cell subpopulation may emerge through selection by the host defense or therapeutic interventions.[18] Thus the tumor heterogeneity may account for the development of subclones with enhanced metastatic capacities or for different susceptibility to destruction by cytotoxic agents.

Large squamous cell carcinomas, which are all solid tumors, have to be considered—especially in advanced stages—as a very heterogenous tumor cell population with varying biologic behaviors. Since these inoperable tumors of the head and neck are treated predominantly by chemotherapeutic drugs and radiation, it is of greatest interest to determine the reactivity of each tumor cell composition individually before the onset of treatment. Although in vitro cultured cell lines might be considered as homogeneous, squamous carcinoma cell lines from different locations of the head and neck are morphologically very heterogeneous, in spite of intensive cloning.[19,20]

It has been shown that the complex cell populations in squamous cell carcinomas can be efficiently resolved by multiparametric flow cytometry.[4] This is important to obtain quick and reliable results for individual pretherapeutic cytotoxic drug testing in solid tumors.

The present experiments show that histologically, uniformly graded squamous carcinoma cell lines of the head and neck vary with respect to their susceptibility of cytotoxic active agents. The high esterase-positive cells representing the cancer cells showed high responses toward cyclophosphamide, dactinomycin, and aclarubicin for the larynx cancer line (HLaC 78; Fig. 1). High killing was seen as well in the hypopharyngeal line (FaDu) with cyclophosphamide and in the larynx line (HLaC 79) with aclarubicin. No increase of dead/vital cell ratios was observed, however, in the squamous cell carcinoma lines SCC-15 and SCC-25 of the tongue and of the parotid gland (HPaC 79; Fig. 2), although the number of vital cells decreased significantly (Fig. 5). This demonstrates that the dead cells quickly autolyze, which is not the case in the HLaC 78 and 79 and FaDu cell lines (Figs. 1 and 3). These differences demonstrate the hetergeneous behavior of squamous carcinoma cell lines from the head and neck to cell killing mediated through cytotoxic drugs. The nonsquamous carcinoma cell lines (3T3 and BC-1) reacted strongly after incubation with cyclophosphamide and aclarubicin; this corresponds well to the clinical susceptibilities of mesenchymal tumors from similar origins.

The results show that a substantial number of in vitro cultured squamous carcinoma cell lines are not suitable for cytotoxic drug screening, since resistance and sensitivity patterns do not correspond to the clinical chemotherapeutic responses. It seems important, therefore, to analyze individual patient tumors by multiparametric flow cytometry, as we did originally.[4] The cell lines seem to be of great interest, however, for the investigation of biochemical changes in drug resistant surviving cells. The changes in volume, esterase activity, and especially in pH show that the surviving cells are considerably altered in their metabolic pattern by the cytotoxic drugs. Thus, the multiparametric flow cytometry has a high potential in the identification and characterization of biochemically abnormal subpopulations of tumor cells under chemotherapy.

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