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Culture of Clonogenic Cells from Various Human Tumors: Drug Sensitivity Assay

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Abstract—*Cells obtained from human solid tumor biopsies and from ascites of various histological origin were cultured in soft agar. Forty-six out of 72 malignant biopsy specimens, consisting mainly of ovarian and breast adenocarcinoma, were grown successfully. Cloning efficiencies ranged from* 0.001 *to* 1%. *In a few cases large variations occurred in the different specimens from the same patient. The cells in the colonies had the same morphological characteristics as those of the original tumor. The soft agar assay was used to study the chemotherapeutic response of ovarian tumors.*

INTRODUCTION

THE EXISTENCE of stem cells in the normal self-renewing tissues such as bone marrow or intestinal epithelium has led to the concept of stem cells in malignant tumors [1]. The clonogenic cells in solid tumors may represent the stem cells, the key replicative units of a malignant tumor. They possess an unlimited proliferative capacity, they can repopulate a depleted tumor after treatment and they can disseminate from the tumor to form metastases. Thus stem cells constitute the crucial cell population of tumors regarding the prognosis and treatment of cancers. In the past ten years, many efforts have been made to estimate the human tumor stem cell pool using *in vitro* culture techniques. Hamburger and Salmon [2] and Courtenay and Mills [3] proposed *in vitro* soft agar colony assays in which tumor cells are selected in a three-dimensional semi-solid medium according to their ability to proliferate and to produce colonies. This bioassay had previously been applied for numerous hematopoietic and solid tumors [4–6]. Hopefully they can be applied to predict the effectiveness of chemotherapeutic agents in patients [7–10].

In this paper we report our initial experience with the human tumor stem cell assay applied to various solid tumors, primarily melanomas and ovarian and breast cancers, to determine *in vitro* sensitivity to chemotherapeutic agents.

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MATERIALS AND METHODS

Ninety-seven biopsy specimens from 90 patients were received in the laboratory. Fifty-nine patients with breast cancers had infiltrating ductal carcinoma. Twenty-four ovarian cancers were adenocarcinomas of epithelial type. Biopsies from 11 patients with melanomas, 1 colon adenocarcinoma, 1 hypernephroma and 1 leiomyosarcoma were also included in this study. All biopsies were from patients at an advanced stage of disease.

Preparation of cell suspension

Tumor biopsy specimens were collected in Eagle's Minimum Essential Medium (MEM) with 10% heat-inactivated fetal calf serum (HIFCS) and immediately dissociated. In a few experiments biopsies were stored at 4°C for 24–48 hr before dissociation. Tumor specimens were mechanically dissociated with a scalpel and needles. Cell clumps were eliminated by passage of the cellular suspension through 100 mesh metallic grids and cloth filters. These cells and those of malignant effusion were washed once in MEM-HIFCS 10% and centrifuged at 200 *g*. Cell viability was estimated in a hemocytometer using the trypan blue exclusion test.

Soft agar assay

Tumor cells were cultured in a two-layer agar assay as described by Hamburger and Salmon [2]. Briefly, a 1-ml lower layer of 0.5% agar (Bacto) in enriched MacCoy's 5A medium (Gibco) was poured into a 35-mm plastic Petri dish and

allowed to solidify at room temperature. Five hundred thousand cells suspended in 1 ml of 0.3% agar in CMRL 1066 medium (Gibco), with 15% HIFCS, feeder compounds and antibiotics, were poured onto the 0.5% agar underlayer. All assays were carried out in triplicate. After solidification of agar the Petri dishes were inspected under an inverted microscope to detect and count clumps. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The cloning efficiency (CE) is the ratio between the number of colonies formed and the number of viable cells plated.

Counting and identification of colonies

Cultures were examined with a Leitz inverted-phase microscope at 50, 125 and 250× magnification. Colony counting was undertaken 21 days after plating: aggregates of 50 or more cells were scored as colonies. To study the colony morphology, the two-layer agar containing colonies was fixed and embedded in paraffin. Thin sections obtained were stained with hematoxylin and eosin. They were examined by pathologists and compared with the section from the original tumor. For ultrastructural studies, colonies were plucked at random with fine-tipped micropipettes, fixed in 2% glutaraldehyde, embedded in Epon and cut in semi-thin or ultrathin sections.

Drug sensitivity test

Drugs were added to the cell suspension in 0.3% agar before solidification of the upper layer; therefore cells were continuously exposed to the drugs.

For any given drug, Alberts *et al.* [9] proposed 10% plasma drug concentration × time product $\left(\frac{C \times T}{10}\right)$ to estimate the minimum *in vitro* drug concentration. According to this estimation and the appropriate *in vivo* $C \times T$ values [9], we calculated the minimum *in vitro* concentration required to a time of 21 days by dividing $\frac{C \times T}{10}$ by a factor of 500. In each drug assay three concentrations covering the minimum *in vitro* concentration were used.

The following anticancer drugs were tested: adriamycin, cisplatinum, melphalan and bleomycin. In addition, ellipticinium (2-methyl 9-hydroxy ellipticine acetate), a drug investigated at the Institut Gustave Roussy [11, 12], was studied.

RESULTS

Obtention of cellular suspensions

Using a mechanical dissociation, cellular suspensions were obtained with a percentage of viability ranging from 0 to 70% (median value, 15%) and cellular yields varying between 0.5×10^6 and 15×10^6 cells/g (median value, 3×10^6 cells/g). The success of the dissociation step depended on the origin of the tumoral specimen. In breast cancers in particular, primary tumors were very difficult to disaggregate, while metastatic nodes were easy. Sixty-three out of 94 biopsy specimens were plated in soft agar: 37/59 breast, 18/22 ovarian, 10/12 melanoma, 1/1 colon, 1/1 hypernephroma and 1/1 leiomyosarcoma. Therefore about 30% of the tumoral specimens could not be tested in the human tumor clonogenic assay for the following reasons: insufficient weight or size, hardness of the tumoral specimen, absence of viability or low cellular yield of the cellular suspension.

Colony growth

In ovarian neoplasms growing clusters were observed within 5–10 days of plating and the colonies could be scored within 18 and 21 days. However, in the case of breast adenocarcinoma, as colonies grew slowly, scoring and identification were made about 28 days following plating. Sixteen out of 22 ovarian specimens produced colonies in soft agar, with a CE value ranging between 0.001 and 1% (Table 1). For breast cancer, in the range of 10^4–5×10^5 plated cells there was a linear relationship between the number of nucleated cells plated and the number of colonies (Fig. 1). This result indicating that no cellular feeder effect occurred in this range of cell concentration made it possible to test the action of chemotherapeutic agents on the tumoral cells.

Successful growth was achieved with 15/29 primary and 6/8 metastatic nodes of breast adeno-

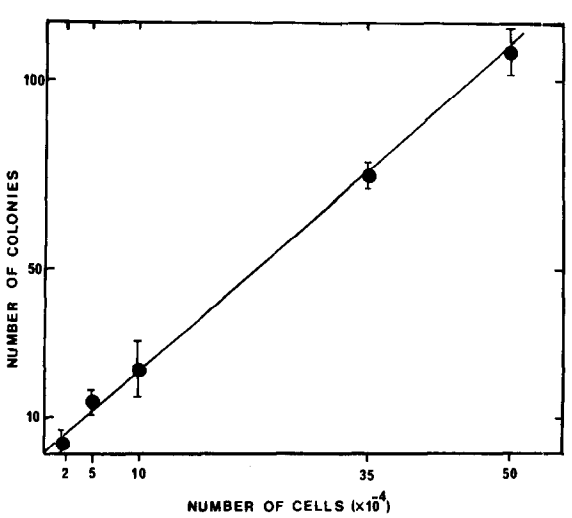

Fig. 1. Number of colonies as a function of plated nucleated cells (breast cancer).

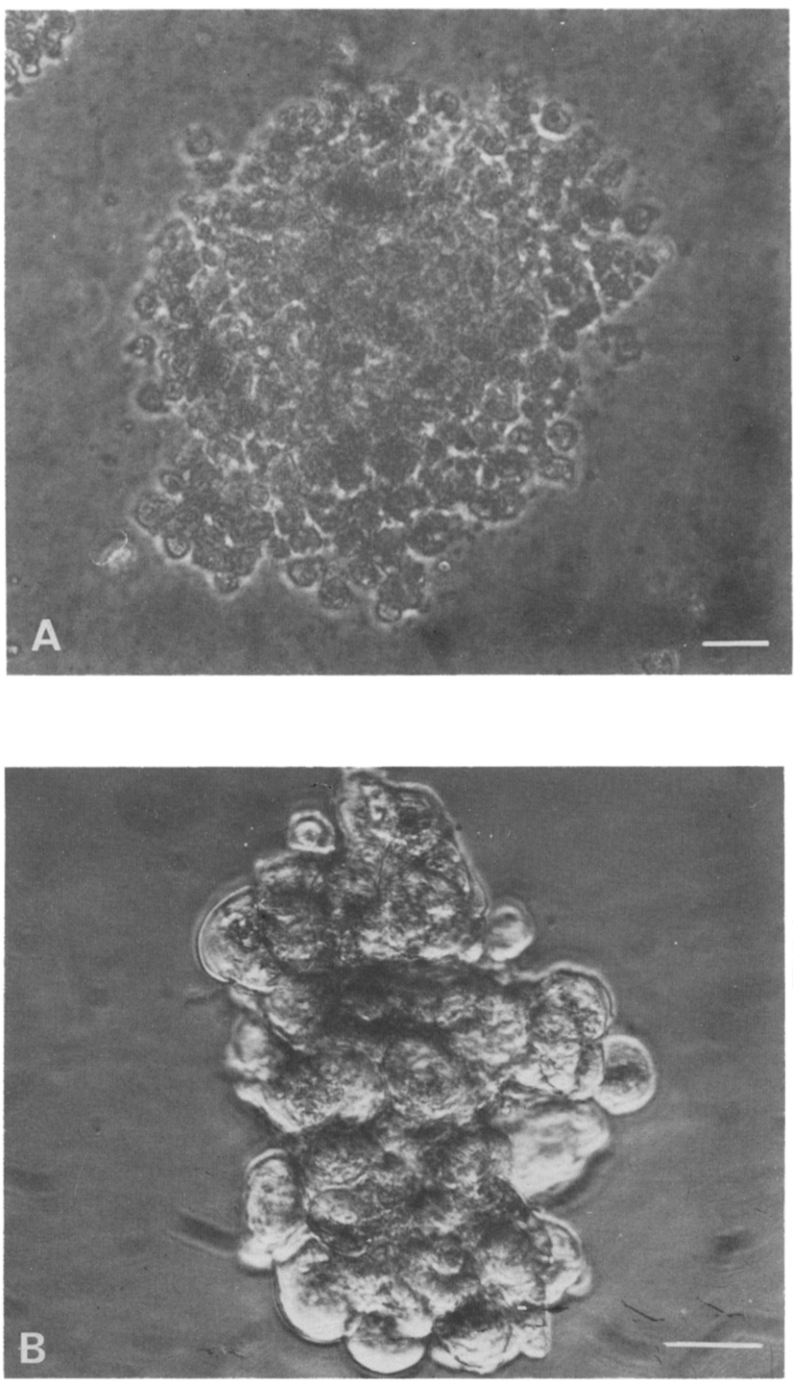

Fig. 2. Typical colonies growing in soft agar from: (A) breast adenocarcinoma; (B) ovarian adenocarcinoma (the bar represents 20 μm*).*

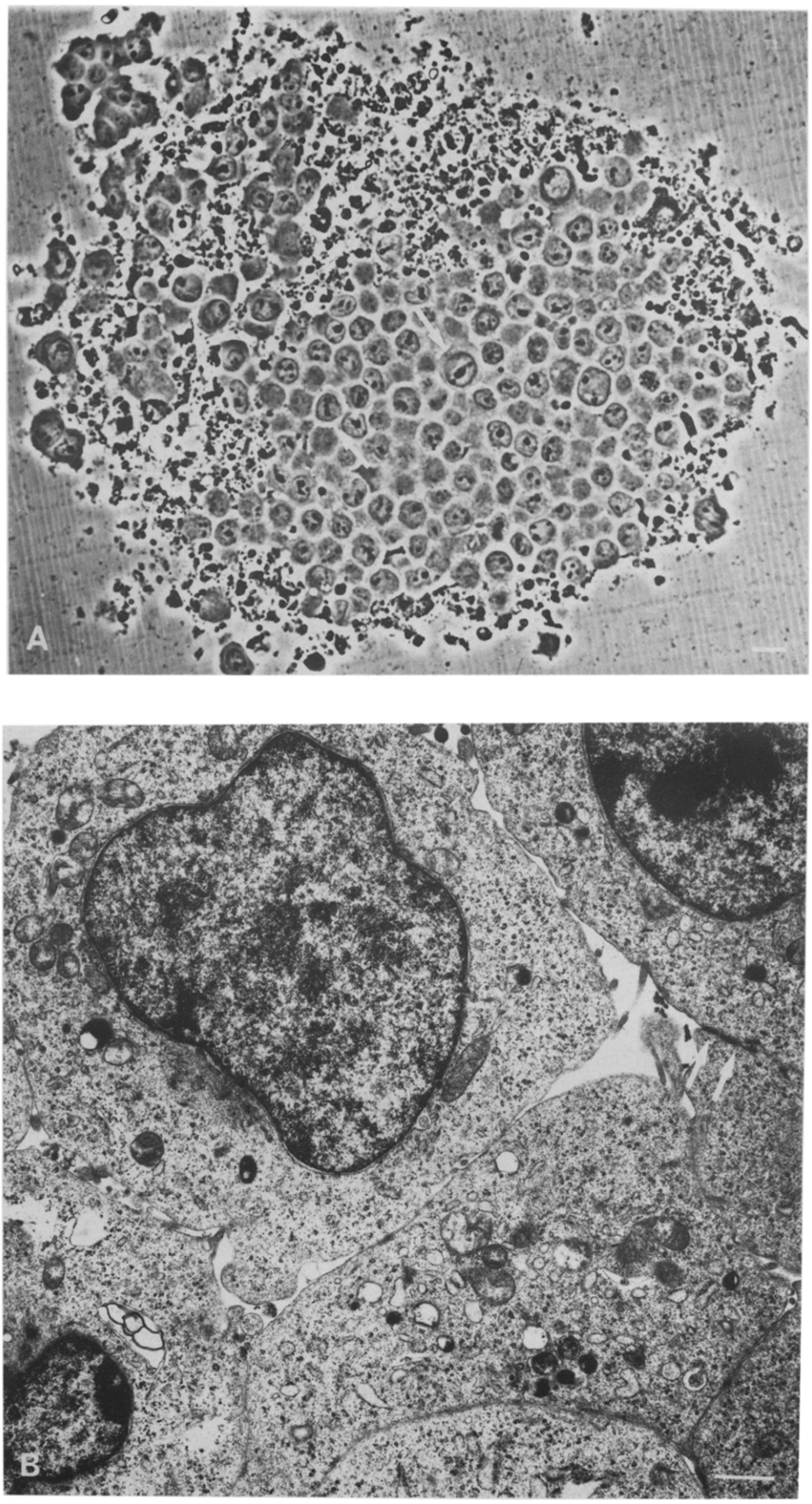

Fig. 3. Sections of ovarian adenocarcinoma colonies plucked from soft agar: (A) semi-thin section viewed through the light microscope. Single arrow indicates mitotic cells (the bar represents 10 μm*); (B) ultrathin section observed under the electron microscope. Double arrow indicates cellular junction (the bar represents* 1 μm*).*

Table 1. Human tumor clonogenic assay for various human neoplasms

Type of tumor	No. of tumors with positive culture/ No. of tumors plated	Cloning efficiency (%)
Ovarian adenocarcinoma		
Primary tumor	13/18 (4/6)*	0.001–1.0
Ascitic effusions	3/4	
Breast adenocarcinoma		
Primary tumor	15/29 (3/5)*	0.005–0.1
Metastatic lymph nodes	6/8 (1/1)†	0.05–0.5
Melanoma		
Primary tumor	5/7 (3/4)*	0.003–0.1
Metastatic nodes	2/3 (1/1)†	
Colonic adenocarcinoma	1/1	0.03
Adult hypernephroma	1/1†	0.015
Leiomyosarcoma	0/1	

Overall success rate was 67%. Unless specified (* or †), tumoral cells were plated within 4 hr of surgical exeresis.
*Tumor biopsies kept in MEM-HICFS 10% at 4°C for 24 or 48 hr before plating.
†Cultures carried out from dissociated cells frozen at -70°C.

carcinoma. The CE values for the primary tumor varied between 0.005 and 0.1% and for the malignant nodes between 0.05 and 0.5%. The results obtained with other tumors (melanoma, colon, adenocarcinoma, hypernephroma and leiomyosarcoma) are presented in Table 1. The overall yield of success rate for growth of the various plated tumors was 67% (46/72). It is only 47% (46/97) if we consider all the tumoral specimens received in the laboratory.

In some experiments the biopsy specimen was kept in MEM-HICFS 10% at 4°C for 24–48 hr and then plated. In these conditions, 4 out of 6 ovarian, 3 out of 5 breast adenocarcinomas and 3 out of 4 melanomas were grown successfully. Their CE values fell in the same range as those obtained from freshly plated tumors. Although only a few experiments were carried out under such conditions, these data suggest that tumor can be stored at 4°C for 24 or 48 hr in cell culture medium.

Primary tumors and metastases were compared from the same patients. In one case of breast adenocarcinoma the clonogenicities of tumoral cells from primary tumor and those from lymph metastatic nodes were compared and found equal to 0.005 and 0.1% respectively. Similarly, in the case of a bilateral ovarian adenocarcinoma CE values were compared: 0.04% for the left ovary and 0.01% for the right one. These two examples suggest that primary tumor and metastatic extensions differ with respect to their growth in the assay.

Characteristics and identification of colonies

Figures 2A and B show typical colonies growing in soft agar. Colonies appeared within 15–30 days of plating, depending on tumor type, and consisted of from 50 to several hundred cells. Ovarian colonies were observed to be larger than breast colonies.

The colonies plucked from agar, embedded in paraffin and stained by hematoxylin–eosin showed identical histological characteristics with those of original tumor. For example, Fig. 3 shows preparations of colonies obtained from an ovarian adenocarcinoma of epithelial type. When observed through the light microscope in semi-thin sections, some mitoses could be seen; at the periphery of the colony necrotic cells were observed, indicating cellular rearrangement (Fig. 3A). Under the electron microscope desmosome-like structures, characteristics of epithelial origin, were observed (Fig. 3B).

Drug sensitivity studies

Adriamycin, cisplatinum and cyclophosphamide, which are currently combined in a EORTC regimen for ovarian cancers, were tested in this bioassay. Melphalan was used instead of cyclophosphamide, which requires bioactivation. Bleomycin and ellipticinium were also tested. Cultures of biopsy specimens from 7 patients produced various numbers of colonies but never less than 50 per control Petri dish. For drug assay the plated tumoral cells were continuously

exposed to each drug during 21 days in concentrations ranging from 1×10^{-1} to 2×10^{-2} μg/ml. Drug responses varied from patient to patient (Figs 4A, B and C). Seventy percent of the assays did not give any *in vitro* response, indicating tumoral resistance to the drugs. In two patients bleomycin caused an important reduction in the survival of clonogenic cells (patients 2 and 6). Two cases responded to adriamycin (patients 1 and 2), while cisplatinum led to one complete and one partial response (patients 2 and 6 respectively).

DISCUSSION

Human tumor clonogenic assay in semi-solid medium has been proposed previously to predict

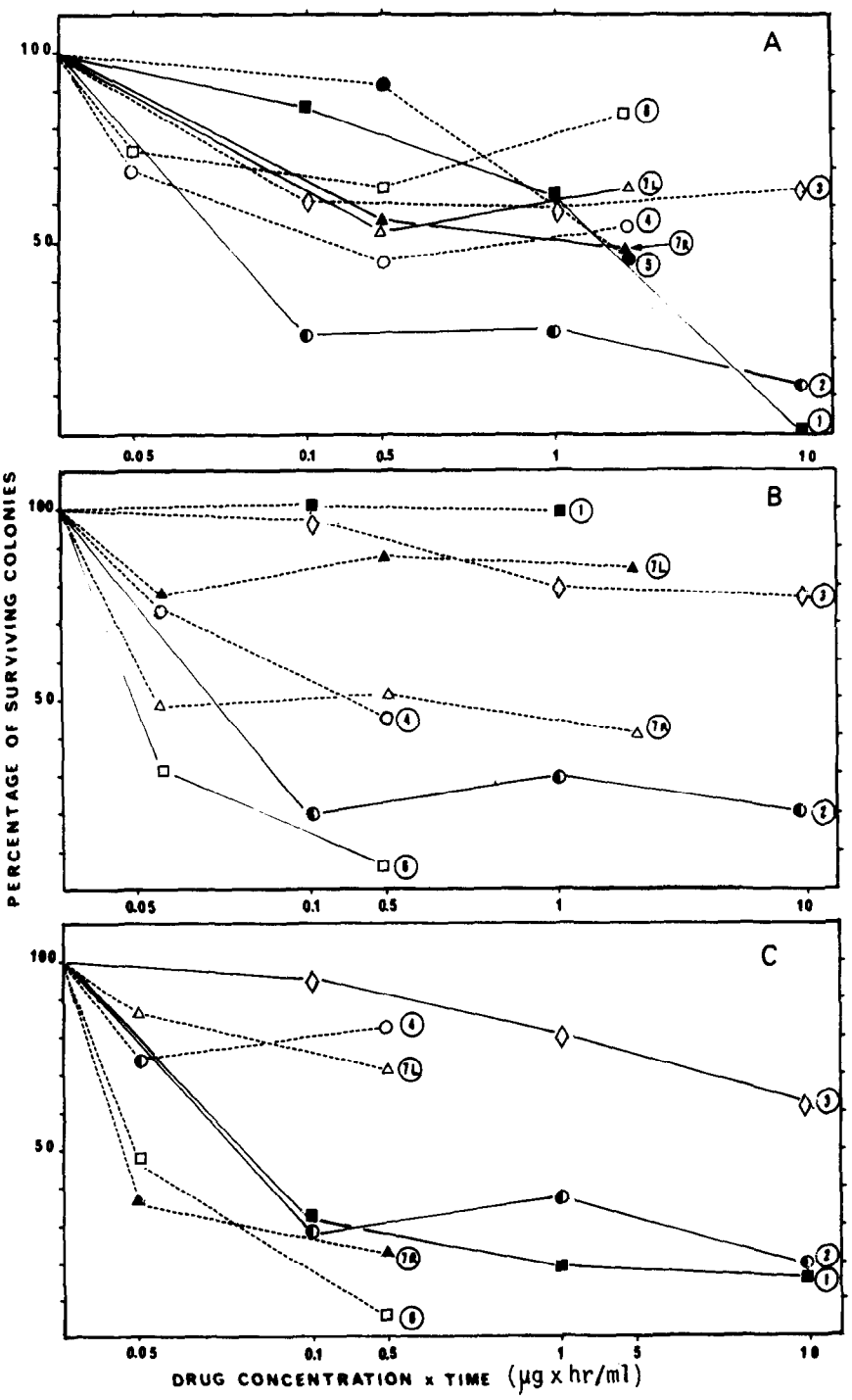

Fig. 4. Action of drugs on colony-forming cells in ovarian carcinomas. Percentage of surviving colonies is plotted as a function of the C × T product of drug, expressed in μ × hr/ml. Biopsy specimens from ovarian carcinoma treated with (A) adriamycin; (B) cisplatinum; (C) ellipticinium (1), melphalan (2, 3) or bleomycin (4, 6, 7_L, 7_R). 7_L and 7_R represent the left and right ovary of patient No. 7.

response or lack of response of a patient's tumor to drugs. In this paper we used the clonogenic assay technique described by Hamburger and Salmon [2] to study methodology problems and the potential of this assay technique.

About 30% of the pathological specimens received could not be plated in soft agar: the small size or hard consistency of the tumoral material submitted to mechanical disaggregation provided an insufficient number of cells. The problem of mechanical disaggregation appeared crucial in the case of breast cancers, where the dissociation success rate is very low in primary tumors as compared with metastatic nodes. In general, disaggregation of the tumors was the limiting step of this bioassay. It has been reported that enzymatic disaggregation gives improved yield of viable and functional cells [13, 14]. Applied to solid tumors, and especially to breast adenocarcinoma, this technique might considerably improve the overall success of growth.

Growth of tumor clonogenic cells was considered to have occurred if at least 5 colonies developed in the Petri dish, corresponding to a CE value of 10^{-5} after plating 5×10^5 cells. Although most of the positive cultures exhibited a higher CE value (median value, 5×10^{-3}), this parameter constitutes a first limitation of the bioassay. It must be pointed out that cloning efficiencies obtained using the Hamburger and Salmon bioassay [2] are lower than those obtained with the soft agar method of Courtenay and Mills [3]. Nutrients and culture conditions explain these differences, as reported in a recent study [15]. Sixty-three out of 94 tumor specimens grew in semi-solid agar, i.e. 67% of growth success rate, in agreement with published data [8]. For primary tumors, ovarian adenocarcinomas grew better in semi-solid agar than did breast carcinomas (16/22 vs 21/37). For the same histological type of cancer, the measured CE value obtained differed from patient to patient. Moreover, in a given patient with ovarian or breast cancer, a higher CE value was obtained from the metastasis than from the primary tumor. Disaggregation procedure as discussed above and/or the tumor heterogeneity may explain these data. Higher numbers of colonies from malignant nodes may have resulted from the selection advantages of some subpopulations arising from the primary tumor and constituting metastatic nodes after proliferation.

For all histological types of cancer, colony size is usually related to CE value. Hence a short doubling time of clonogenic cells is required if colonies are to be obtained within 15–21 days of culture in agar. This would explain, in part, the failure of 33% of the tumor specimens to grow. In addition, although only a few cases are reported in this paper (4/5), high CE values were found in patients with a poor prognosis and short life span. Relationships between prognosis and growth in clonogenic assay have recently been reported [16]. Our observations suggest that clonogenic assay could be applied mainly to highly proliferating and malignant tumors. Therefore it would be of great interest to measure the doubling time of the tumor [17] in the initial tumoral cell suspension.

As confirmed by histological studies, colonies developed in the soft agar assay consisted of cells similar to those from the tumor biopsy specimen. Nevertheless, even if cells of colonies exhibited some of the pathological criteria of malignancy (mean width of nuclei, nuclei/cytoplasm ratio, mitotic activity, etc.) as a consequence of the soft agar procedure, the absence of tissue organization and the distribution of tumoral cells inside a stroma did not allow us to conclude that clonogenic cells represented malignant proliferating cells of the tumor. Further biological studies, such as hetero-transplantation of the colonies in the nude mouse, are required to confirm this point.

The *in vitro* response of their tumors to chemotherapeutic drugs was studied in seven patients with ovarian cancers. The *in vitro–in vivo* correlations are not yet available since a second laparotomy is normally undertaken one year after ovariectomy. Nevertheless, three features of the results should be noted: (a) in a given tumor, differences in responses to various drugs indicated the heterogeneity of the clonogenic cells with respect to one phenotypic character, i.e. drug sensitivity. In addition, plots of percentage of surviving colonies as a function of drug concentration often exhibited a biphasic curve: a marked decrease followed by a plateau. This reflects the presence of at least two subpopulations of clonogenic cells, one sensitive and the other resistant; (b) in one patient (patient No. 7) and for a single drug, a difference in chemotherapeutic responses between the primary tumor and metastatis was observed; (c) for a given type of cancer, large variations in the *in vitro* drug response of patients were observed.

Despite all the limitations we stressed above (technical difficulties, low cloning efficiencies), human tumor clonogenic assay could probably be used to help the physician in his choice of chemotherapeutics agents: prediction of resistance can be made to within 95% correlation [7, 18], thus saving patients from exposure to needless and toxic drugs. In addition, human tumor clonogenic assay is of valuable interest in the case of highly proliferating and potentially metastasizing cancer. Many technical improvements are

required before this bioassay can be used in the follow-up of pharmacological treatments of tumors. Thus, at present, its application to predict the suitability of treatment seems to us impossible for routine use on technical and theoretical grounds.

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