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The Soft Agar Clonogenicity and Characterization of Cells Obtained from Human Solid Tumors by Mechanical and Enzymatic Means

Harry K. Slocum, Zlatko P. Pavelic, Peter M. Kanter, Norma J. Nowak, and Youcef M. Rustum

Department of Experimental Therapeutics and Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, NY 14263, USA

Summary. *A two-step procedure for releasing cells from solid tumors has been applied to specimens of human melanoma, sarcoma, lung, colon, and breast carcinoma. The first population released mechanically has been compared with the population subsequently released enzymatically in tests of dye exclusion, ribonucleoside triposphate pool sizes, intactness of DNA, and clonogenicity in soft agar. While greater numbers of dye-excluding cells are released in the enzymatic step, and these cells have higher ribonucleoside triphosphate pools and more intact DNA, both populations contain clonogenic cells in approximately equal numbers.*

Several semisolid media were employed in tests of clonogenicity, and all methods employing an agar underlayer appeared satisfactory and approximately equivalent in cloning efficiency. The methyl cellulose upper layer system facilitated implanting of pooled colonies into nude mice, which resulted in growth in the nude host and marked increase in cloning efficiency when the cells were replanted into soft agar-methyl cellulose plates.

A comparison of four different areas of individual tumor specimens was made with cells released enzymatically and measuring cell yield, dye exclusion, ATP pool size, and uptake and metabolism of 5-fluoropyrimidines. Only relatively small variations were seen from one area to the next, with trypan blue exclusion exhibiting the least variation, and metabolism of fluorinated pyrimidines showing the most.

Introduction

A test to predict the chemotherapeutic sensitivity of human malignant tumors would be of great benefit to patients, enabling oncologists to select effective agents for individuals. The search for such a test to put chemotherapy firmly on a rational basis has been conducted largely on an empirical basis itself. Early attempts to identify drug sensitivities of individual tumors employed slices or minces of tumor material either directly, or after short-term culture [2, 3]. In the long run these tests did not provide the needed predictive correlations. It is apparent that an understanding of the characteristics of the cells comprising solid tumors and definition of the determinants of drug action are the keys to the design of predictive tests and rational design of chemotherapeutic protocols. Recent progress toward obtaining information in this area, however, has been hampered by the difficulties associated with obtaining large numbers of viable cells from human solid tumor tissue for study.

We have recently developed a two-step method for obtaining large numbers of viable cells from human solid tumor specimens [13, 14], in which a mechanical disaggregation step is followed by an enzymatic disaggregation step. This method was developed to maximize cell yield and minimize cell damage, and provides cell suspensions suitable for characterization through cytology, culture in semisolid media [8, 9, 13, 14] and athymic mice [9], karyology [16], flow cytometry, determination of ribonucleoside triphosphate pools [13, 14], intactness of DNA, and uptake and metabolism of radiolabeled drugs. In this report we present data comparing cells released mechanically and enzymatically from human melanoma, sarcoma, and carcinoma of the lung, colon, and breast, and compare clonogenicity in five semisolid media systems.

Material and Methods

Solid Tumor Disaggregation. A two-step disaggregation procedure as described previously [13, 14] was employed, and is outlined below.

Reprint requests should be addressed to H. K. Slocum

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Mechanical Disaggregation. Mechanical disaggregation consisted of slicing the tumor tissue into 0.5-mm slices with a Stadie-Riggs microtome [15] (A. H. Thomas Co., Philadelphia, PA), followed by brief mincing with crossed scalpel blades in ice-cold RPMI 1640 medium with 10% heat-inactivated fetal bovine serum. Cells thus freed were separated from the tissue fragments by pouring over a 100 mesh stainless steel screen (E−C Apparatus, St Petersburg, FL). Remaining tissue was gently returned to the petri dish and the mincing procedure repeated until cell release was no longer significant (one or two cycles were usually required). Cells obtained in this manner were pooled and were considered mechanically released. Remaining tissue was treated enzymatically as described below.

Enzymatic Disaggregation. After initial trials with various proteolytic enzymes in various combinations and sequences, we selected a mixture of 0.8% collagenase II (Worthington Biochemicals, Freehold, NJ) and 0.002% deoxyribonuclease I (Sigma Chemical Co., St Louis, MO) dissolved in RPMI 1640 medium with 10% inactivated fetal bovine serum (Grand Island Biological Co., Grand Island, NY). The tissue mince was exposed to enzyme for 2 h at 37° C in a humidified atmosphere of 5% CO_2−95% air. Freed cells were collected by pouring through a 100 mesh screen as described for mechanical disaggregation.

Cell Counting. Cells were counted after dilution in saline in the presence of a final concentration of 0.1% trypan blue (Direct Blue 14 from MC1B, Norwood, OH). The lesser of five fields (0.5 mm^3) or 100 cells of each type (excluding or failing to exclude dye) were counted in an improved Neubauer hemacytometer chamber, employing 400× magnification.

Histopathology and Cytology. A small sample of tumor material was placed in 10% buffered formalin before disaggregation and kept for histopathology. Slides of cell suspensions were prepared by means of cytospin centrifuge (Shandon Southern) and fixed with hairspray [10] for Papanicoulou staining [6] or air-dried for Wright's staining [6]. The non-specific esterase method of Yam et al. [17] was used to achieve differential staining of granulocytes and monocytes.

Ribonucleoside Triphospohate Pool Profiles. High-pressure liquid chromatographic analysis of neutralized perchloric acid extracts was accomplished as described by Rustum et al. [12].

Clonogenicity in Semisolid Media. Two assays employing soft agar, two employing methyl cellulose and soft agar, and one employing methyl cellulose alone were used. Briefly the soft agar assay of Pluznik and Sachs [11] employed a 0.5% agar lower layer in Eagle's medium with 10% fetal bovine serum beneath an upper layer of 0.3% agar in the same medium. The soft agar method of Hamburger and Salmon [4], without conditioned medium, employed a 0.5% agar lower layer in McCoy's 5A medium with 10% fetal bovine serum and an upper layer of 0.3% agar in enriched CMRL 1066 medium with 15% horse serum (Flow Laboratories, Rockville, MD). The methyl cellulose-soft agar system of Buick et al. [1] employed an upper layer of 0.8% methyl cellulose (A4M from Dow Chemical Co., Midland, MI) in McCoy's 5A medium with 10% fetal bovine serum, and a lower layer of 0.5% agar in the same medium. The methyl cellulose-soft agar system of Pavelic consisted of an upper layer of 1 ml, as described for the system of Buick et al. [1], and a lower layer of 2.5 ml 0.5% agar in Eagle's medium with 10% fetal bovine serum. All plating methods routinely utilized 500,000 cells inoculated in the upper layer in 35 × 10-mm plastic petri dishes. Colonies of 30 or more cells were counted 2−3 weeks after plating, at 200× magnification with a microscope equipped with inverted phase optics.

DNA Damage. DNA damage was assessed employing a new technique developed by Kanter and Schwartz [5]. The assay is based on differential molar fluorescence of bisbenzamide in association with single- and double-stranded DNA after partial alkaline denaturation. Briefly, neutralized alkali-denatured cell extracts were sonicated in the presence of bisbenzamide (0.25 μg/ml final), 0.01 *M* ethylenediaminetetraacetic acid, and 0.04% lauryl sarcosinate, in 0.05 *M* phosphate buffer (pH 7.4). Duplex/total DNA ratios were then determined from the fluorescence (excitation 353 nm emission 451 nm) compared with that of completely denatured or native DNA.

Transplantation of Colonies into Nude Mice. Single colonies (approximately 10^3 cells) or pools of colonies (3.5×10^5−10^6 cells) were taken from methyl cellulose upper layers, rinsed in antibiotic solution (penicillin and streptomycin), and resuspended in RPMI 1640 cell culture medium. These were injected subcutaneously into the groin and axilla of 10-week-old homozygous nude female mice of BALB/c background (Harlan/Sprague-Dawley Animal Resources, Madison, WI) [9].

Drug Uptake and Metabolism. The uptake and metabolism of 6-^{3}H-5-fluorouracil (1.67 Ci/mmole, 10 μCi/ml) and 6-^{3}H-5-fluorodeoxyuridine (1.67 Ci/mmole, 10 μCi/ml) were determined by 1-h incubation of the cell suspension at 37° C in the presence of radiolabel, followed by saline washing at 4° C, cold perchloric acid extraction, and high-pressure liquid chromatographic analysis as described by Rustum et al. [12].

Results

Disaggregation

The yield of dye-excluding cells by enzymatic release is displayed in Fig. 1, with comparison to the median values for each disease type by mechanical release. Mechanical disaggregation resulted in variable cell yields from individual tumors, even with in a disease type. The median yields were highest for lung tumors (39×10^6 total cells/g) and melanoma (29×10^6 total cells/g), and lowest for sarcoma (13×10^6 total cells/g). However, only a small fraction of the cells released mechanically were able to exclude dye. The median dye-excluding fraction was highest for lung tumors (28%), and 8%−10% for the other tumor types.

Enzymatic treatment yielded cell suspensions consisting primarily of dye-excluding cells [14]. Median values ranged from 79% viability for melanoma to 90%−95% for the other tumor types. There was wide variation in cell yield even within a disease type, as was the case with mechanical release. The number of dye-excluding cells obtained, however, was generally much greater from the enzymatic than from the mechanical step in the disaggregation procedure. As can be seen from Table 1, the probability of obtaining a yield of 20 million dye-excluding cells per gram of tissue is much higher if the enzymatic technique is employed than if only mechanical treatment is used.

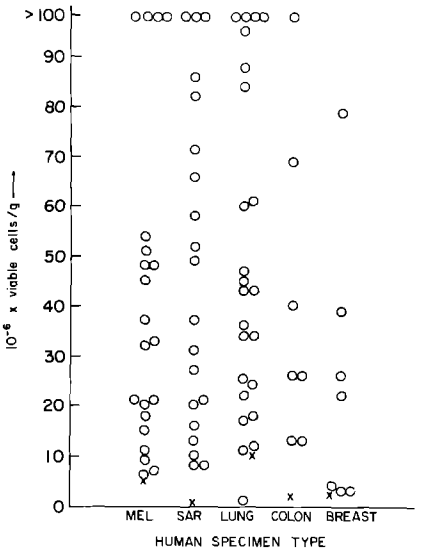

Fig. 1. Specimens of human solid tumors were disaggregated by the two-step mechanical and enzymatic procedure. The number of trypan blue-excluding cells obtained in each step was determined. The number obtained enzymatically is shown for each case by the *open circles*, and the median number obtained mechanically for all cases in the disease type is shown by the ×. (○) = C II; (×) = Median value mechanical

Ribonucleoside Triphosphate Pools

Ribonucleoside triphosphate pools for cells released mechanically and enzymatically from melanoma and lung tumors are displayed in Table 2. The pools are generally higher in the enzymatically released cells, especially in melanoma. In lung tumor cell suspensions, pools were generally reduced, especially the pool of ATP. Sarcoma specimens seldom yielded enough cells by mechanical disaggregation to allow comparison with the enzymatically released cells. When the comparison was possible, the pools in the enzymatically released cells were higher, showing the same pattern observed in melanoma and lung [14].

Culture in Semisolid Media

Rat or mouse red blood cells, or conditioned media from human or mouse cultures, or delayed plating of cells did not increase colony growth in our application of Pluznik and Sachs system [8, 9]. A comparison of the Pluznik and Sachs system with the others tested is displayed in Table 3. It can be seen that the overall success rate, i.e., the probability of achieving growth of 30 or more colonies on a plate, is similar for all the methods tested, with the exception of the Ogawa system. The median cloning efficiency seen on a

Table 1. Comparison of the yield of viable tumor cells disaggregated by mechanical and enzymatic methods from human tissues[a]

Tumor type	Fraction of experiments in which yield > 20 × 10^6 viable cells/g	
	Mechanical	Enzymatic
Melanoma	13/80 (16%)	14/21 (67%)
Sarcoma	1/43 (2%)	15/20 (75%)
Pulmonary cancer	10/66 (15%)	19/24 (79%)
Colon cancer	0/10 (0%)	5/6 (85%)
Breast cancer	0/9 (0%)	4/7 (57%)
Overall	24/208 (12%)	57/78 (73%)

[a] Human tumor specimens were disaggregated by the two-step mechanical and enzymatic procedures, and the number of dye-excluding cells determined. The table shows the probability of obtaining more than 20 million cells per gram of tumor for any specimen of a given disease

Table 2. Ribonucleoside triphosphate pools of cells released mechanically (M) and enzymatically (E) from human solid tumors[a]

Tumor	Specimen code	M or E	nmoles/10^7 total cells			
			CTP	UTP	ATP	GTP
Melanoma	12–3	M	–	–	0.732	–
		E	0.462	1.36	13.9	3.10
	12–6	M	–	0.524	3.98	0.693
		E	–	2.63	23.6	4.40
	11–5	M	–	0.113	0.571	0.110
		E	0.333	2.08	14.7	2.28
	10–6	M	–	–	–	–
		E	0.369	1.75	14.2	3.31
	8–3	M	–	–	–	–
		E	1.733	–	14.6	5.23
	8–2	M	–	–	0.128	–
		E	0.252	0.441	17.7	1.17
	10–1	M	–	–	0.622	–
		E	–	0.600	11.0	1.69
	10–2	M	0.988	0.458	2.60	0.219
		E	1.04	2.72	14.6	1.87
Lung	7–1	M	–	–	0.138	–
		E	–	–	1.64	–
	7–3	M	–	–	1.52	0.306
		E	–	–	0.917	0.237
	7–6	M	0.441	0.658	3.92	1.02
		E	0.402	–	3.52	1.39
	6–4	M	0.121	0.572	1.36	0.423
		E	0.362	0.500	6.57	2.78

[a] The ribonucleoside triphosphate pools of cells released by mechanical and enzymatic means were measured in neutralized perchloric acid extracts by high-pressure liquid chromatography [12]

successful plate was also similar overall, with either agar-agar or methyl cellulose-agar systems. In the Ogawa system cells penetrated the lower layer and contacted the plastic substrate, and either failed to grow, or only a few fibroblast-like colonies were formed.

The methyl cellulose-agar systems facilitated manipulation of colonies for implantation into nude mice [9]. Single colonies failed to produce tumors in nude mice, but colonies pooled from a single plate totalling $3.5-10 \times 10^6$ cells did produce tumors from human melanoma, sarcoma, and ovarian carcinoma [9]. Enzymatic disaggregation and replating of these cells was accomplished with melanoma and ovarian carcinoma cells. The clonogenicity in the case of melanoma was $88.7 \pm 6.1/10^5$ cells plated before passage, and $3,880 \pm 967$ after, a 43-fold increase. Clonogenicity of the ovarian carcinoma increased from 131 ± 35 to $1,510 \pm 75$, an 11-fold difference.

Clonogenicity of Mechanically and Enzymatically Released Cells

In the soft agar system of Pluznik and Sachs we compared the clonogenicity of cells released by the mechanical and enzymatic treatments. In spite of marked differences in the trypan blue exclusion in the two types of suspension, we found no significant differences in clonogenicity in any tumor type [14]. In Table 4, median clonogenicities and success rates are

Table 3. Clonogenicity of cells for human solid tumors in five semisolid media systems[a]

	A[b]	B	C	D	E
Median colonies per 500,000 cells plated[c]	80 ± 28	158 ± 9	127 ± 37	0	93 ± 26
Success rate[d]	16/23	12/22	12/20	0/15	11/14
%	70	55	60	0	79

[a] The clonogenicity of cells released enzymatically from specimens of human melanoma, sarcoma, and carcinoma of the lung, ovary, breast, and colon was determined in five systems employing semisolid media
[b] A, system of Pluznik and Sachs [11]; B, system of Hamburger and Salmon [4]; C, system of Buick et al. [1]; D, system of Ogawa et al. [7]; E, system of Pavelic (see *Materials and Methods*)
[c] Median number of colonies ± standard deviation, on all plates growing > 30 colonies, over all disease types
[d] Number of specimens growing > 30 colonies/total of specimens plated

Table 4. Clonogenicity of cells obtained from human solid tumors by mechanical (M) and enzymatic (E) means

Disease	Suspension type[a]	Success rate	Median clonogenicity[b]
Melanoma	M	14/22	130 ± 30
	E	18/22	162 ± 15
Sarcoma	M	8/16	31 ± 11
	E	12/16	74 ± 25
Lung carcinoma	M	11/16	87 ± 40
	E	12/16	56 ± 10
Breast carcinoma	M	5/8	178 ± 20
	E	5/8	85 ± 30
Colon carcinoma	M	6/11	57 ± 16
	E	9/11	224 ± 65

[a] Cell suspensions were prepared by the two-step mechanical and enzymatic procedure, and plated in the soft agar system of Pavelic as described in *Methods*
[b] Colonies of > 30 cells/500,000 cells plated, ± standard deviation

Table 5. Average molecular weight of DNA obtained from cells disaggregated from human solid tumor tissue by mechanical (M) and enzymatic (E) means

Patient	Diagnosis	$10^{-8} \times M_R$ alkaline undwinding unit	
		M	E
S. D.	Squamous cell Ca	< 2	6.2
P. L.	Squamous cell Ca	< 2	15.0
J. K.	Adenocarcinoma	6.5	2.4
M. H.	Mesothelioma	< 2	20.0
O. D.	Carcinoid	3.1	5.5
G. L.	Melanoma	< 2	2.4
C. P.	Adenocarcinoma	< 2	2.4
S. E.	Melanoma	< 2	< 2
H. A.	Adenocarcinoma	6.4	6.8
A. A.	Sarcoma	3.9	7.7
S. E.	Adenocarcinoma	< 2	2.9
L1210	Mouse, ascites	15	–

[a] Average molecular weight of DNA from cells obtained by mechanical and enzymatic means was determined by the method of Kanter and Schwartz [5]

shown for the five major tumor types. The medians are slightly higher for enzymatically released cells in the case of melanoma, sarcoma, and colon, and lower in lung and breast carcinomas. The probability of obtaining > 30 colonies on a plate, however, is generally greater with cells released enzymatically. Cell suspensions from normal human skin, lymph node, liver, colon, and spleen failed to form colonies in the soft agar assay.

DNA Damage

The average molecular weight of DNA obtained from cells released mechanically and enzymatically is displayed in Table 5. The value obtained for murine leukemia L1210 is included for reference. The cells released in the enzymatic step revealed less damage to their DNA in ten of eleven cases. The median value for enzymatically released cells was 5.5×10^8 unwinding units compared to $< 2 \times 10^8$ for mechanically released cells. The average molecular weight is greater than 2×10^8 for only four of eleven cases in mechanically released, as against ten of eleven cases for enzymatically released cells.

Comparison of Different Areas of an Individual Tumor Specimen

We compared the cell yields obtained by mechanical and enzymatic release from different areas of a given specimen by cutting the specimen into four pieces and disaggregating each separately. We also compared the ability of the resultant cell suspensions to exclude trypan blue, their ribonucleoside triphosphate pools, and their uptake and metabolism of radiolabeled fluorinated pyrimidines. In Table 6 the yield, dye exclusion, and ATP pool sizes of the cells released enzymatically from melanoma, sarcoma, and lung tumor are displayed. While some variation is apparent, the values are generally similar from the four different areas. Table 7 displays the uptake of radiolabeled 5-fluorouracil (FU) and 5-fluoro-2′-deoxyuridine (FUdR) into acid-soluble and acid-insoluble intracellular material, and metabolism of

Table 6. Yield and characteristics[a] of cells released from four different areas of individual tumors

Code	Disease	$10^{-6} \times$ viable cell yield/g	Dye exclusion (%)	ATP nmoles/10^7 cells
J0813 −1	Sarcoma	72	94	9.50
−2		128	93	5.91
−3		101	96	8.17
−4		121	91	5.45
M0430 −1	Sarcoma	37	83	25.5
−2		49	84	25.1
−3		31	63	5.0
−4		42	79	17.5
S0605 −1	Sarcoma	49	97	n.d.[b]
−2		39	96	n.d.
−3		13	92	n.d.
−4		42	93	n.d.
H0730 −1	Lung Ca	145	62	1.19
−2		185	63	2.29
−3		145	53	2.30
−4		132	58	1.10
R0605 −1	Lung Ca	410	88	11.0
−2		324	90	9.2
−3		303	89	13.0
−4		306	92	8.5
T0730 −1	Melanoma	175	91	4.49
−2		230	93	2.15
−3		96	93	3.58
−4		107	92	3.69

[a] The yield, trypan blue exclusion, and ATP pool sizes of cells obtained enzymatically from four different areas of individual tumors specimens were determined as described in *Methods*

[b] n.d., not determined

Table 7. Uptake and metabolism of fluorinated pyrimidines by cells released from four different areas of individual tumors[a]

Code	Disease	FU (pmole/10^7 cells)			FUdR (pmole/10^7 cells)		
		Acid-soluble	FdUMP	Acid-insoluble	Acid-soluble	FdUMP	Acid-insoluble
J0813 −1	Sarcoma	54	0.7	0.80	139	62	0.45
−2		40	3.01	0.77	98	99	0.57
−3		36	1.65	0.68	201	114	0.43
−4		24	2.20	0.46	104	84	0.38
S0605 −1	Sarcoma	26	0.35	3.23	44	14	4.8
−2		29	0.64	2.64	86	18	5.2
−3		39	0.59	8.18	112	14	16
−4		28	1.07	4.39	86	13	8.9
H0730 −1	Lung Ca	262	9.74	0.16	247	130	0.27
−2		243	11.9	0.20	275	144	0.25
−3		246	8.29	0.19	187	97	0.18
−4		235	2.51	0.45	259	183	0.49
R0605 −1	Lung Ca	21	0.50	2.1	47	29	3.7
−2		28	1.15	3.1	61	41	6.0
−3		39	0.73	3.3	100	30	6.9
−4		24	0.62	2.9	68	19	5.1

[a] The uptake and metabolism of radiolabelled fluorinated pyrimidines was determined as described by Rustum et al. [12] in cells obtained from four different areas of individual tumor specimens

each compound to 5-fluoro-2'-deoxyuridine monophosphate (FdUMP). Although there was considerable variation from one tumor to the next, even within the same tumor type, variation from one area of a given specimen to another was much less. Of the 24 comparisons made of areas within individual tumors, 16 showed maximum values not exceeding twice the minimum value. In eight comparisons, variations from 2.5- to 4.7-fold were observed. Four of the wide variations were in acid-insoluble incorporation of FU and FUdR, and the one instance of greatest variation (4.7-fold) was in FdUMP formation from FU by cells from different areas of a single specimen of lung carcinoma.

Discussion

The disaggregation method described yields cells from solid tumors sequentially, in two steps. The first step is purely mechanical, freeing cells available without enzymatic action. The second step, applied to remaining tissue, frees cells enzymatically. The two suspensions may represent different populations, more or less firmly bound into the tissue matrix. It is not presently known what portion of the total cells in the tumor are released by these methods, what changes in the cells are created by removing them from the tissue matrix, or whether the two types of suspensions contain different quantities of the cells most dangerous to the patient.

It is apparent that while the number of cells released from a tumor specimen is quite variable, more cells are generally available through use of enzymes than by simple mechanical treatment. Also, these suspensions are characterized by much higher numbers of dye-excluding cells. Studies requiring suspensions free of trypan blue-staining cells clearly require the cells available enzymatically. Enzymatically released cells also have higher ribonucleoside triphosphate pools, and their DNA displays less damage than the DNA of those obtained mechanically.

In other respects, however, the suspensions generated by the two steps are qualitatively similar. They are indistinguishable in cytological profile [14]. While karyotyping is seldom possible with mechanically released cells, when it is successful similar chromosomal aberrations are found in the cells released mechanically and in those released enzymatically [16]. Apparently both suspensions contain important populations of cells, as clonogenicity in semisolid media is similar in both types of suspension in all disease types we have studied thus far. The questions of whether there will be differences in in vitro drug sensitivity or accuracy in prediction of in vivo sensitivity are the subjects of current investigation.

Apparently several systems of semisolid media meet the growth requirements of clonogenic cells, provided the underlayer is able to prevent the cells from sinking through to the plastic substrate. Also, while we were unable to identify a supplement to increase cloning efficiency, passage of colonies through the nude mouse markedly increased clonogenicity. This is, of course, at least partly due to the selection of clonogenic cells before implantation. Growth in the nude mouse combined with cloning in semisolid media may provide a means of amplifying the clonogenic population to allow study of mechanisms of drug resistance and sensitivity.

Anatomical segregation of important cell populations within human solid tumors would allow the potential for significant sampling error when specimens are taken at surgery and used to produce cell suspensions for study of clonogenicity, or determinants of drug action. Entire populations of important cells might be missed if such segregation exists. We therefore investigated the question of heterogeneity within different regions of the same tumor nodule. Arbitrarily quartering the tumor specimen, of course, is not the ideal way to search for anatomical segregation. We selected this approach because no gross anatomical clues as to the location of possibly segregated populations were available in the human specimen. Cutting the specimen into smaller pieces was not possible because a sufficiently large piece (at least 0.5 g) must be used to obtain sufficient cells for study from each area. A further implication of this size requirement is that small specimens (and thus small tumors) were eliminated from the study at the outset. Keeping in mind these limitations, we cannot conclude that anatomical segregation within human solid tumor does not exist, but we found the four areas of the tumor we examined to be similar in cell yield, dye exclusion, ribonucleoside triphosphate profile, and in most instances drug uptake and metabolism. We are currently expanding the investigation of anatomical segregation, employing cloning in semisolid media, karyology, and flow cytometry. Since these methods examine cells individually, rather than averaging large populations, they may provide more detailed information regarding anatomical segregation, and heterogeneity on a cellular level.

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