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CLINICAL AND PHARMACOLOGICAL IMPLICATIONS OF CANCER CELL DIFFERENTIATION AND HETEROGENEITY *

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Abstract—Various aspects and interrelationships of intra-neoplastic diversity have been investigated at two levels: (1) differences among multiple subpopulations of tumor cells at any point in time (heterogeneity) and (2) variations among these subpopulations as a result of their maturation with time (differentiation). The importance of tumor cell heterogeneity and differentiation in neoplasia has been studied using cancer cells obtained from murine mammary tumors and rhabdomyosarcoma, as well as from patients with carcinoma of the colon. Several cancer cell lines derived from these tumors have been cloned and characterized. Neoplastic cell differentiation has been induced using a polar solvent, *N,N*-dimethylformamide (DMF); the differentiation is evidenced by morphological maturation, conversion of tumor cell markers and cell culture characteristics to those consistent with a more benign phenotype, and loss of tumorigenicity. Striking morphological and biological heterogeneity has been observed in neoplastic subpopulations isolated from a single tumor, including marked variability in growth potential, surface antigens, and sensitivity to chemotherapeutic agents. The biological and pharmacological significance of these findings is discussed from the standpoint of their profound implications for clinical therapy.

That cancer is a dynamic disease, heterogeneous in terms of time and space, has long been appreciated by those who have studied the biology of neoplasia. Thus, many investigators, most prominently Foulds [1] and Klein and Klein [2], have described the changing behavioral patterns of cancer as a function of time, a phenomenon known as progession. Other workers, such as Gray and Pierce [3], Prehn [4], Leighton [5], and more recently Fidler and Kripke [6], have demonstrated the morphological and behavioral heterogeneity among tumor cells of a single neoplasm. Nevertheless, the predominant view of cancer, as exemplified by the experimental systems used among researchers in the therapeutics of cancer, is one of a homogeneous, monotonous, uniform disease. For example, drugs and drug combinations are assessed against relatively few cell lines that have been maintained artificially for many years under laboratory conditions chosen to ensure homogeneity. Although this approach has provided much useful information, one can question whether ignoring the dynamic nature of cancer will ever lead to permanent control of the disease. Our laboratory has developed models to analyze the heterogeneity of cancer with the hope that knowledge of this most basic characteristic of the disease will provide relevant insights into its control. These models are based on two levels of heterogeneity, one which exists at any point in time among multiple subpopulations of tumor cells, and the other that develops with time as tumor cells differentiate. These two levels necessarily are interrelated.

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MATERIALS AND METHODS

Cell lines. Rhabdomyosarcoma cells were obtained from a transplantable tumor (BW10139; Jackson Laboratory, Bar Harbor, ME) passaged in CE/J mice.

Tumor tissue was excised and minced, and cultures were obtained either from cells growing out from small tumor fragments or from tumor mince treated with 0.25% trypsin (Grand Island Biological Co., Grand Island, NY) to obtain single-cell suspensions. Cell suspensions or fragments were cultured in tissue culture dishes (Falcon No. 3001 or No. 3002; Falcon Plastics, Oxnard, CA) that had been treated previously with 2 ml of 0.02% gelatin (Difco, Detroit, MI) for 3 hr at 4° to provide a substratum. Growth medium consisted of Eagle's basal medium supplemented with 15% heat-inactivated horse serum (both from Grand Island Biological Co.) and containing 25 μg gentamicin (Schering Corp., Port Reading, NJ) and 100 units mycostatin (Grand Island Biological Co.) per ml respectively.

Human colon carcinoma cell lines designated DLD-1 and HCT-15 were derived from specimens of adenocarcinomas of human colon removed during the normal course of surgery at Brown University-affiliated hospitals. Informed consent was obtained from these patients. Tumor tissue was debrided of fat and minced thoroughly, the mince was washed three times in Hank's balanced salt solution (HBSS) (Grand Island Biological Co.) and digested by sequential application of three to four 30-min changes of 0.25% trypsin (Grand Island Biological Co.) in HBSS at 23°. The cells were collected at each change of trypsin, washed once in HBSS and placed in 60-mm tissue culture

dishes (Falcon No. 3002, Falcon Plastics). All cultures were grown in RPMI 1640 medium supplemented with 20% heat-inactivated fetal calf serum, 100 units penicillin/ml, 100 μg streptomycin/ml and 2.5 μg fungizone/ml (all from Grand Island Biological Co.). The medium was buffered with 0.075% $NaHCO_3$, 10 μM HEPES and 10 μM tricine (all from Grand Island Biological Co.). The culture medium was decanted and replaced with fresh medium at 2- to 3-day intervals after plating. For routine passage, cells were treated with 0.25% trypsin and then replated in dishes (Falcon No. 3001 or 3002) or flasks (Falcon No. 3013 or 3024). Clones were obtained by selecting colonies growing in soft agar.

Cultures were derived from the first *in vitro* passage of a mammary tumor spontaneously arising in one of our Balb/cfC_3H breeding females. Our Balb/cfC_3H mice are the progeny of a breeding pair obtained in 1969 from the Cancer Research Laboratory, University of California at Berkeley. They carry mouse mammary tumor virus (MMTV). The tumor was excised, minced and treated with 0.25% trypsin (Grand Island Biological Co.) in calcium–magnesium free Earle's balanced salt solution (Grand Island Biological Co.). The resulting cell suspension was plated in Falcon tissue culture flasks (Falcon Plastics). When the culture grew to a monolayer, the cells were harvested with 0.25% trypsin in 0.9% NaCl solution and centrifuged for 5 min at 1400 rev/min in a Sorvall GLC-2 centrifuge. The pellet was resuspended in Waymouth's medium (Grand Island Biological Co.) supplemented with 15% heat-inactivated fetal calf serum (Grand Island Biological Co.) and 2 mg/ml of L-glutamine (Grand Island Biological Co.) and 20 μg/ml of gentamicin (Schering Corp.), and the cells were plated in three 60-mm dishes. Colony isolation was achieved from these secondary cultures through the technique of differential trypsinization. With this method, cells are sparsely seeded in a culture dish. When isolated colonies appear, medium is removed from the dish, and a drop of trypsin is placed on a selected colony. The detached cells are removed with a Pasteur pipet and introduced into fresh medium in a new culture dish. This procedure is then repeated one or two additional times. Clones from soft agar were obtained from cells from the isolated lines.

All cultures were kept at 37° in a humidified atmosphere of 5% CO_2 in air.

Growth in agar. When cultures were grown in semi-solid medium, cells were resuspended in 0.5% agar (Difco) in complete growth medium, and 1 ml of this suspension was layered on a 2-ml base of 1.0% agar in medium in a 35-mm dish (Falcon No. 3001).

Growth rate. For determination of cell doubling times, replicate culture dishes received inocula of 5×10^4 cells, and cells from two dishes were harvested separately each day and counted with a hemacytometer. Results were plotted on semilogarithmic paper, and doubling times were calculated from the curves.

Cloning efficiencies. Cloning efficiencies on plastic dishes were determined by counting the colonies present in a dish 1 week after seeding. Cloning efficiencies in agar were determined by counting the colonies in a dish 2 weeks after the culture was initiated. In both cases one observer counted colonies in duplicate dishes by complete scanning of each dish with the aid of a light microscope.

Saturation densities. These data were obtained from the results of experiments performed to determine doubling times. In confluent dishes, the numbers of cells present that determined the plateau regions of the growth curves, provided the saturation densities, defined as cells/cm^2.

Karyotype analysis. Exponentially growing cultures were incubated with 0.2 μg/ml of colcemid (Grand Island Biological Co.) for 5 hr. Cells were harvested and chromosome preparations were made according to standard techniques. The preparations were observed under a phase microscope and at least 25 metaphases were analyzed for each cell type.

Induction experiments. *N,N*-Dimethylformamide (DMF; Fisher Scientific Co., Fair Lawn, NJ) was added to cultures to give a final concentration of 0.8% (v/v) DMF in the colon carcinoma cell growth medium, or 1% (v/v) in the growth media for the mouse rhabdomyosarcoma or mammary tumor cells. Treated cells used in experiments were passaged at least twice in the presence of DMF and were cultured 10 or more days in drug-containing medium. Treated cells were seeded in agar medium containing 0.8 or 1% DMF.

Mammary tumor virus antigen assay. Cells were harvested from confluent monolayers by treatment at 37° with 0.25% trypsin; from this point the procedure was carried out at 4° by keeping the cells on ice and incubating in a refrigerator. The cells were harvested as described above. The suspension was centrifuged at 250 *g* for 5 min and the pellet was washed with Tris-buffered saline (TBS, 0.25 M Tris–hydroxymethyl aminomethane, Sigma Chemical Co., St. Louis, MO, in 0.9% NaCl; pH 7.8). The washing was repeated twice, and the cells were resuspended in 0.1 ml of a 1:20 dilution in TBS of rabbit anti-C3H MMTV antiserum kindly donated by Dr. J. Gruber, Viral Oncology, National Institutes of Health. The suspension was agitated every 5–10 min for 30 min, the cells were centrifuged, and the pellet was washed three times with TBS. The cells were then resuspended in 0.1 ml of fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories, Downingtown, PA) diluted 1:4 with TBS. After mixing for 30 min, the cells were centrifuged, and the pellet was washed three times with TBS. The cells were resuspended in three to five drops of Tris–glycerine (9 parts glycerol: 1 part TBS) and added dropwise to a microscope equipped for fluorescence microscopy. A negative test was one in which no fluorescent cells were seen. In control experiments, nonimmunized rabbit serum was used.

Colon tumor cell markers. Membrane-associated carcinoembryonic antigen (CEA) was detected by the classical indirect fluorescent antibody assay. Goat anti-CEA was provided by Dr. Hans Hansen of Hoffmann–LaRoche Laboratories (Nutley, NJ). Tumor and normal colonic mucoprotein antigens (CMA) were also detected by the indirect immunofluorescence antibody assay. The rabbit anti-CMAs were provided by our collaborators, Drs. Frederick Miller and David Gold of the State University of New York—Stony Brook. The blood group reactivity of cultured cells was evaluated with the standard sheep red cell adherence assay. Cells were grown in LabTek slide culture chambers (LabTek Products, Napierville, IL) and were fixed when subconfluent in 10% buffered neutral formalin. Antisera to blood groups A or B, or ulex reagent (for detection of

blood group O) were used to assay the blood group determinants of the fixed cells, as indicated by adherence of sheep red cells present in the incubation mixture.

Mice. Strain Balb/cfC_3H male mice, aged 2–4 months, were purchased from the Cancer Research laboratory, University of California, Berkeley. CE/J mice were obtained from the Jackson Laboratory. Athymic, nude mice bearing the nu/nu genotype on an outbred Swiss background are bred and maintained in the Roger Williams General Hospital Animal Care Facility.

Tumorigenicity studies. Female Balb/cfC_3H mice, male CE/J mice, and nude mice, 6–10 weeks of age, were used as hosts for cell injections. Cells in culture were trypsinized and centrifuged as above. The pellet was washed twice and suspended in 0.9% NaCl solution to give the appropriate density. Usually each mouse received a subcutaneous injection of 1×10^6 viable (trypan-blue excluding) cells in a volume of 0.2 ml. In studies designed to compare the *in vivo* growth capacity of the lines, mice received subcutaneous inocula of 10^6, 10^5, 10^4 or 10^3 cells.

Histology. Morphological studies of tumors were carried out on the original tumors and tumors from the derived cell lines. All tissue was obtained fresh and was fixed immediately in Bouin's solution. Histological sections were stained with hematoxyline and eosin, Mayer's mucicarmine, Periodic Acid-Schiff (PAS) and alcian blue (at pH 1).

In vivo *sensitivity studies.* Each mouse was ear-tagged and treated individually. There were at least ten mice per group. The mice were assigned to treatment groups on a random basis using a random number table. Mice receiving the various drugs and doses were appropriately caged. The mice were examined twice weekly for the appearance and growth of tumors. The tumors were measured in two dimensions with calipers. The slope of the growth rate was computed from the curve generated by plotting mean surface area on the ordinate, and weeks on the abscissa, on plain graph paper.

All drugs were given intraperitoneally once a week for 4 weeks starting either 2 days post subcutaneous injection of tumor cells or immediately after the appearance of palpable tumors. Methotrexate (MTX; Lederle Laboratories, Pearl River, NY) was used at a weekly dose of 50, 25 or 10 mg/kg, 5-fluorouracil (FU; Hoffmann-La Roche Labs) at 50, 25 or 10 mg/kg, and cyclophosphamide (CY; Mead Johnson Laboratories, Evansville, IN) at 100, 50 or 25 mg/kg. Control mice received 0.9% NaCl solution.

All mice surviving for 3–4 months after tumor cell injections were autopsied and all organs were examined grossly for visible metastatic foci. A mouse was scored as positive for metastasis if at least one nodule was found in the lungs or visceral organs.

In vitro *sensitivity studies.* On day 1, replicate tissue culture dishes (Falcon no. 3001, Falcon Plastics) were each inoculated with 5×10^4 cells in the appropriate growth medium. Cells were harvested by trypsin treatment from two of the dishes on day 2 and counted with a hemacytometer; this determined the number of cells per dish at the time of drug addition. Methotrexate, fluorouracil or actinomycin D was added in concentrations ranging from 10^{-4} to 10^{-11} M to other cultures on day 2. Drug-treated and control cultures were harvested by trypsin treatment on day 5, and the numbers of cells were determined. The number of doublings for non-treated cells was calculated from the cell number in control dishes on days 2 and 5. The drug concentration required to inhibit the number of doublings in the 72 hr period by 50 per cent (ID_{50}) was then calculated from the dose–response curve, in which cell numbers were plotted against molar drug concentrations.

RESULTS

DMF induction of differentiation in rhabdomyosarcoma cells. When cultured in 1% DMF for periods of 2–7 days, the morphological characteristics of the rhabdomyosarcoma cells were altered from an irregular, triangular appearance to one of bipolar cells that formed swirl patterns in dense cultures. This changed appearance was seen in all of the cells and was not accompanied by loss of viability or proliferative capacity. The cell culture characteristics of the induced cells were also changed. Thus, the treated cells showed an *in vitro* doubling time of 22 hr, as compared to 12 hr for the control cells. The saturation density of DMF-treated cells was 2.9×10^4 cells/cm^2 compared to 1.1×10^5 cells/cm^2 for the controls. There was no colony growth of treated cells in soft agar, whereas an inoculum of 300 control cells had a cloning efficiency of 48 per cent. Most significantly, the tumorigenicity of treated cells was reduced markedly. Thus, twenty-one of twenty-one mice receiving injections of 10^6 untreated cells died of tumors in an average time of 18 days, whereas only three of seventeen mice injected with identical numbers of DMF-treated cells developed tumors in 6 months. All these changes are reversible because cultivation of treated cells in DMF-free medium results in the reappearance of pre-induction characteristics. Therefore, the effects of DMF treatment are not due to selection of variant cell types.

Evidence that these changes indicate differentiation along the myogenic pathway has recently been obtained in collaboration with our colleague, Dr. John Coleman [7]. DMF-treated cells have been found by histochemical methods to have an increased level of glycogen phosphorylase, a muscle-related enzyme [8]. More significantly, although untreated cells have undetectable levels of creatinine phosphokinase (CPK), as measured by a linked-dehydrogenase reaction [8], treated rhabdomyosarcoma cells have appreciable levels (0.4 units/mg of protein) of this enzyme, comparable to those found in fusing chicken embryonic myoblasts. Since creatine phosphokinase is only found in muscle and brain, its presence in DMF-treated cells strongly suggests that muscle differentiation has been achieved.

DMF induction of differentiation in human colon carcinoma cells. One cell line for these studies was established from a moderately to poorly differentiated adenocarcinoma of the sigmoid colon resected from a 45-year-old caucasian man. The parent line, DLD-1, has been cloned into two distinct subpopulations; cloning was performed in soft agar and the clones were designated A and D. Another tumor cell-line was derived from a well to moderately differentiated adenocarcinoma of the sigmoid colon in a 64-year-old caucasian man, and is designated HCT-15. The characteristics of these cell lines are shown in Table 1.

Table 1. Effect of DMF on properties of human colon carcinoma cells

Cell line	Untreated characteristics						DMF-Treated characteristics					
	Morphology		Karyotype	CEA‡,§	TCMA‡,‖	Ulex¶	Morphology		Karyotype	CEA‡,§	TCMA‡,‖	Ulex¶
	Cell colonies*	Tumor†					Cell colonies*	Tumor				
DLD-1 parent	Circumscribed	M-P	Mostly 45–46 Few hyperploid	1+	1:16, 68%	2+	Branched	None	Mostly 45–46 Few hyperploid	3+	1:4, 55%	1+
DLD-1 clone A	Circumscribed	P	Hyperploid	1+	1:16, 40%	3+	Branched	None	Hyperploid	3+	1:4, 27%	1+
DLD-1 clone D	Circumscribed	M	45–46	2+	1:16, 75%	2+	Branched	None	45–46	4+	Neat, 64%	1+
HCT 15	Circumscribed	W-M	45–46	1+	1:4, 40%	3+	Branched	None	45–46	2+	1:4, 30%	1+

* Epithelial.
† M = moderately differentiated, P = poorly differentiated, W = well differentiated.
‡ Flourescent antibody assay.
§ Carcinoembryonic antigen, intensity of fluorescence.
‖ Tumor colonic mucoprotein antigen, endpoint dilution, percentage cells fluorescing.
¶ Sheep red cell adherence assay, 1:8 dilution of Ulex reagent; H-gene determinant.

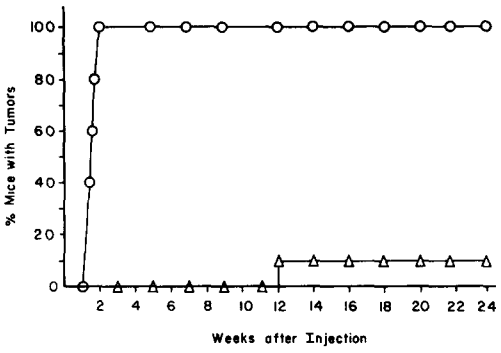

Fig. 1. DLD-1 cells from subconfluent dishes were removed by trypsinization, washed twice with 0.9% NaCl solution, and then were resuspended in the NaCl solution. Each nude mouse, 6- to 10-weeks-old, received an intraperitoneal injection of 1 million trypan blue-excluding cells. Twenty mice were inoculated with untreated DLD-1 cells (○), and ten mice were inoculated with DLD-1 cells previously cultured in the presence of 0.8% *N,N*-dimethylformamide for at least 7 days (△).

Treatment of all four cell lines with 0.8% DMF for 5 days resulted in morphological differentiation *in vitro*, characterized by a change from circumscribed colonies to branched colonies. Cell culture characteristics were also altered, with an increase in doubling times from 20 ± 2 hr to 47 ± 5 hr, a 200–300 per cent decrease in saturation density, and a complete loss of clonogenicity in soft agar. Tumorigenicity studies have so far only been completed for DLD-1. Although twenty of twenty athymic nude mice injected with 10^6 untreated DLD-1 cells developed tumors in 10–14 days, only one of ten mice inoculated with DMF-treated cells developed a tumor after a prolonged latency period of 80 days (Fig. 1). Since tumors developed in two of two mice injected with 10^5 untreated cells and four of four injected with 2×10^5 untreated cells, a 10^6 cell inoculum is well above the threshold dose necessary to produce tumors (Table 2).

Three markers were chosen to monitor the changes in differentiation of human colon cancer cells exposed to DMF. As indicated in Table 1, all three markers changed in the direction of a more differentiated phenotype. Thus, reactivity of treated cells with anti-carcinoembryonic antigen (CEA) antibody was increased in accordance with many reports [9–15] that an increase in CEA production correlates with a higher degree of differentiation of human colon tumors and derived cell lines. In addition, reactivity with antisera to the tumor-associated, colonic mucoprotein antigen (CMA) described by Gold and Miller [16] was less in DMF-treated than in control cells. We have also found that this decrease in reactivity with the anti-tumor CMA is accompanied by an increase in reactivity with the anti-normal CMA, also described by Gold and Miller [16]. Finally, reactivity of the treated cells with the Ulex reagent, used for detecting the H-gene-specified blood group determinant, was decreased by DMF treatment. Experiments are in progress to determine whether this loss is due to re-expression of the blood groups appropriate to the patients from whom these tumors were obtained.

The effects of DMF on human colon cancer cells are fully reversible, even after 6 months of exposure to the inducer. Furthermore, DMF has no effect on viability or proliferative capacity of the colon cancer cells in monolayer culture. Similar effects were observed with DMF-treated rhabdomyosarcoma cells. Therefore, selection of variant cell types by DMF cannot be the explanation for our results.

Heterogeneity of tumor cells from a single mammary tumor. Our experiments were initiated from a single, autochthonous Balb/cfC_3H mammary tumor placed in a monolayer culture. From this culture, which contained numerous morphologically and karyotypically distinct cells, we isolated four subpopulations by a variety of cell culture and separation methods. A fifth subpopulation was isolated by culturing cells from a metastatic lung nodule growing in a mouse bearing the tenth *in vivo* passage of the parent tumor. These five subpopulations obtained from a single mammary tumor have been characterized in a variety of ways (Table 3). They differ markedly from each other in every way except one: they all produce tumors upon subcutaneous injection into syngeneic mice. Their tumorigenicity varies in potency, however, from 100 per cent in 4 weeks to 75 per cent in 20 weeks.

The lines have been maintained *in vitro* for 18 months and, in general, have remained stable in their properties. There has been a tendency for decreased doubling times in the 68H line, and, rarely, cultures of both 68H and 168 lines will contain morphological variants. Cultures established from tumors produced *in vivo* by each of the lines are identical to the isolated lines. One exception is the tumor produced by re-inoculating 68H cells which, in addition to cells typical of that line, also contains several morphological variants. These variants are derived from the 68H population, not from the host, as is shown by antigenic and karyotypic analysis. Clones of lines 66, 67 and 168 have been obtained from soft agar. The properties of these clones are identical to those of their respective

Table 2. Tumorigenicity of cultured human colon carcinoma (DLD-1) cells

Treatment	Inoculum No. of cells	Proportion of nude mice with tumors	Latency period (days)
None	1×10^5	2/2	60
None	2×10^5	4/4	30–50
None	1×10^6	20/20	10–14
DMF†	1×10^6	1/10	80

* Observation period, 6 months.

† Cells were cultured in growth medium containing 0.8% *N,N*-dimethylformamide (DMF) for at least 1 week prior to subcutaneous inoculation.

Table 3. Characteristics of subpopulations from a single mouse mammary tumor

Cell line	Modal karyotype (range)	Morphology *in vitro*	Doubling time *in vitro* (hr)	Cloning efficiency in agar/10^5 cells (%)	MMTV surface antigen	Tumor potential/10^5 cells
66	39 (39–41)	Spindle	12	0.5	Negative	75%, 3–8 weeks
67	74 (70–80)	Spindle, lattice	21	19	Negative	100%, 4–6 weeks
168	41 (39–41)	Fibroblastic	17	33	Negative	100%, 4 weeks
68H	130 (110–140)	Epithelial	40	0.1	Strongly positive	75%, 8–20 weeks
4.10	69 (39 ⩾ 100)	Epithelial	15	0	Low, variable	100%, 4 weeks

parents. Lines 68H and 4.10 do not grow in soft agar and, therefore, cannot be cloned by this technique.

Since the five lines have been derived from an MMTV-associated tumor, it is interesting that only one of them, 68H, expresses MMTV surface antigen as detected by a fluorescent antibody technique using anti-MMTV rabbit antiserum. Recently, however, we have shown that the other four subpopulations can be induced to express that antigen by treatment with 5-iodo-2′-deoxyuridine (IUdR). They also express MMTV antigen after exposure to DMF.

Heterogeneity of tumor cells from a single human colon carcinoma. In order to determine whether the phenomenon of tumor heterogeneity applies to human neoplasms, we utilized our colon carcinoma line DLD-1 and its derivative clones, A and D. The patient's tumor, from which this cell line was derived, was a moderately to poorly differentiated adenocarcinoma. Tumors produced by inoculation of DLD-1 cells into athymic, nude mice are characterized by a very similar histology. In nude mice, clone A cells produce poorly differentiated colon carcinomas, and clone D cells produce moderately differentiated colon carcinomas. Clone D cells show a diploid karyotype, whereas clone A cells are hyperploid. The parent DLD-1 line in an early culture passage was predominantly diploid, but a few hyperploid karyotypes were observed. The parent line has always contained two distinct, epithelial cell types. Therefore, the original tumor was heterogeneous, with clone A and D cells being representative of the two subpopulations identified morphologically, histologically and karyotypically in the patient's tumor and the derived DLD-1 line.

Cells from the A and D cloned populations were seeded in soft agar to provide ten recloned subpopulations from each parent clone type. In each case, nine of ten isolated clones produced cultures morphologically identical to the A or D cell type. One A reclone and one D relcone were morphologically different from A or D cells respectively. These variant reclones were analyzed for karyotypes, and the histologies of the tumors obtained after their injection into nude mice were characterized. Representative A or D reclones were randomly selected, and were subjected to the same analysis. These reclones were identical to A or D in karyotype and histology. The variant reclone of A, however, had a diploid karyotype, whereas the variant D relcone produced a poorly differentiated tumor in the nude mouse host. Thus, one of ten A reclones differed from clone A both karyotypically and morphologically, whereas one of ten D reclones differed from clone D both morphologically and in the histology of the tumor it produced.

This spectrum of cultured human colon carcinoma clones and reclones from a single tumor provides a unique model for investigating various aspects of intraneoplastic diversity. Of cardinal importance are the clincal and therapeutic implications of human tumor heterogeneity.

Pharmacological implications of tumor cell heterogeneity. It seems reasonable to postulate that subpopulations of tumor cells within a given neoplasm would exhibit a differential sensitivity to chemotherapeutic agents. Accordingly, we have tested this concept using both the mouse mammary tumor and the human colon carcinoma models. We have tested three of the mammary tumor lines, 68H, 168 and 4.10, for their sensitiv-

Table 4. Relative sensitivity of mammary tumor subpopulations *in vivo* to cyclophosphamide (CY), methotrexate (MTX) and fluorouracil (FU)

Treatment protocols	Cell line	Drugs: CY (25–100 mg/kg)	MTX (10–50 mg/kg)	FU (10–50 mg/kg)
		Per cent regression		
Two days post subcutaneous injection of tumor cells; all doses	68-H	67	28	40
	168	23	4	0
	4.10	0	0	0
		Mean slope of growth rate, (mm^2/week)		
Post appearance of palpable tumors; 50 mg/kg	68-H	0	43	17
	168	24	31	29
	4.10	32	42	46

Table 5. Effect of methotrexate (MTX) and fluorouracil (FU) on *in vitro* growth of mammary tumor cell subpopulations

Cell line	Molar concn of drugs required to reduce doublings by 50 per cent (ID_{50})	
	FU (M)	MTX (M)
68H	3.2×10^{-7}	7.0×10^{-10}
168	1.5×10^{-8}	1.4×10^{-10}
4.10	4.3×10^{-7}	2.9×10^{-10}

ity to cyclophosphamide (CY), methotrexate (MTX) or fluorouracil (FU) both *in vivo* and *in vitro*. These three drugs were selected because of their well-known activity in breast cancer and their clinical use in combination adjuvant chemotherapy. Two *in vivo* protocols were used, one initiating drug treatment 2 days after subcutaneous injection of 10^5 tumor cells and another administering treatment only after appearance of palpable tumors. In both cases the agents were given once a week for 4 weeks. Various drug doses were tested. In the *in vitro* protocol, tumor cells were plated on day 1 and varying concentrations of drugs (MTX or FU) were added on day 2. The numbers of cells in treated and untreated cultures were determined on day 5. The results of all these studies are summarized in Tables 4 and 5. It is evident that these three lines were not uniformly susceptible to the three drugs. Indeed, the differences in sensitivity were quite striking. For example, in the early treatment protocol, a dose of 25 mg/kg of CY/week reduced the outgrowth of 10^5 68H cells from fifteen of thirty mice in the control groups to one of ten mice in the experimental group. No tumors were seen in mice given 50 or 100 mg/kg of CY/week. On the other hand, CY had no effect in reducing the outgrowth of 4.10 cells at any of the three dosage schedules. In addition, line 168 was twenty times more sensitive *in vitro* to FU than was line 68H (ID_{50} values were 1.5×10^{-8} M for 168 and 3.2×10^{-7} M for 68H). By contrast, the early treatment protocol with FU produced regressions in 40 per cent of mice bearing 68H tumors, but no regressions in mice bearing 168 or 4.10 tumors. In the *in vitro* protocol, 168 cells were five times more sensitive to MTX than were 68H cells.

The *in vitro* sensitivities of human colon carcinoma clone A and clone D cells to three antitumor drugs were also determined. Clone D cells were 340 per cent more sensitive to actinomycin D ($ID_{50} = 3.8 \times 10^{-9}$ M) than were clone A cells ($ID_{50} = 1.3 \times 10^{-8}$ M), whereas clone A cells were 400 per cent more sensitive to fluorouracil ($ID_{50} = 1.3 \times 10^{-5}$ M) than were clone D cells ($ID_{50} = 5.2 \times 10^{-5}$ M). Both cell types showed essentially identical sensitivities to methotrexate (ID_{50} values of 2.2×10^{-8} and 2.0×10^{-8} M for clone D and clone A cells respectively). These data provide evidence that human carcinomas are also composed of subpopulations of tumor cells that differ in their sensitivities to antineoplastic agents.

Immunological implications of tumor cell heterogeneity. We are also investigating the relative immunogenicity of these tumor subpopulations. Although our studies are not yet complete, the data indicate that the five mammary tumor subpopulations differ greatly in their ability to elicit, and be affected by, immune responses. Thus, it can be anticipated that their susceptibilities to immunotherapy will also prove to be dissimilar.

DISCUSSION

The spontaneous, biological or chemical induction of differentiation of cancer cells *in vitro* and *in vivo* is currently an area of great interest [17–27]. These observations support the well-known hypothesis of Pierce [28], Markert [29], and others that cancer is a disease of differentiation. We have developed two systems, using murine rhabdomyosarcoma and human colon carcinoma cell lines, in order to study cancer from this perspective. Our goal has been to induce chemically the loss of tumorigenicity with concomitant expression of a better differentiated phenotype. In both systems the method of induction has been the treatment of cultured tumor cells with the polar solvent DMF.

The second level of tumor heterogeneity that we have investigated involves the diversity existing within a tumor cell population at any point in time. For this work we have chosen spontaneously arising mammary tumors in strain Balb/cfC_3H mice and the previously described human colon carcinoma, DLD-1.

Mouse mammary tumors are especially good models for studying heterogeneity. The etiology of these tumors includes the MMTV, as well as endocrine and genetic factors. In her classic description of these neoplasms, Dunn [30] stressed the variability and complexity among and within these tumors. Henderson and Rous [31] demonstrated that mammary tumors contain different subpopulations of neoplastic cells. They implanted tumor fragments from a single tumor into several subcutaneous sites of recipient mice and obtained histologically distinct neoplasms. Sluyser and Van Nie [32] have shown that mammary tumors of strain GR mice contain both estrogen receptor positive and estrogen receptor negative cells. Our data clearly indicate that we have isolated and characterized distinct subpopulations of neoplastic cells from a single mammary tumor. For the following reasons, we conclude that the heterogeneity revealed by our methods was present in the original tumor, which must have contained distinct tumor subpopulations rather than a uniform cell type. The primary culture was morphologically heterogeneous; in fact that was the basis for the selection of the subpopulations. Furthermore, the original tumor contained the full range of karyotypes represented in our isolated lines. In addition, a minority (2–17 per cent) of the cells from the original tumor reacted with anti-MMTV antiserum, consistent with the fact that only one of the sublines is MMTV positive. Although we are unable to determine when the heterogeneity appeared in the development of the neoplasm, it is obvious that its presence has important biological consequences.

Of special importance is the demonstration of analogous results with tumor cells obtained from a human neoplasm, adenocarcinoma of the colon. The ability to dissect and analyze tumor cell heterogeneity in human cancer with our methodology offers the unique opportunity to delve into the clinical and pharmacological implications of tumor substructure.

Clinical implications of cancer cell differentiation and heterogeneity. Several interesting clinical implications may be derived from these new approaches to the

study of human neoplasia. The complex intra-neoplastic interactions responsible for cellular differentiation and heterogeneity must play an important role in such fundamental and poorly understood processes as angiogenesis, invasiveness, ability to metastasize, prolonged latency periods, tendency to elicit immunological or other host reactions, as well as selective sensitivity and development of resistance to currently used chemotherapeutic agents. From the observations reported above, it seems apparent that several of the phenomena described may be manifested at the level of the cell membrane. Accordingly, these interactions may exert important influences on the unique properties of the cell surface that may be intrinsic to the neoplastic process. The opportunity to study new models that provide fresh insight into the biology of neoplasia can redirect our attention to more rational uses of existing antineoplastic agents, as well as stimulate the development of more novel and imaginative methods of therapy.

Induction of tumor differentiation by maturational therapy. Encouraged by the successful induction of differentiation in cultured DLD-1 cells, we are currently evaluating the efficacy of DMF against human colon and breast carcinomas growing subcutaneously in nude mice. Another chemical known for its inducing potential, sodium butyrate, is also being studied. Dosage schedules of 1000 mg/kg were administered intraperitoneally, daily for 10 days, and no toxic manifestations were noted. Although it is too early to consider specific mechanisms of selective tumor cytotoxicity, it is intriguing to speculate that maturational therapy may alter significantly the host–tumor interrelationship. Further studies are in progress, with a view toward appropriate pre-clinical and clinical pharmacological investigations.

New approaches to the therapy of heterogeneous tumors with conventional agents. It has long been recognized that many human neoplasms are histologically heterogeneous. If the basis for this morphological heterogeneity is the existence of discrete subpopulations of neoplastic cells, it is likely that these subpopulations will respond differently to various chemotherapeutic agents. The results presented above are clearly consistent with these concepts. Similar considerations apply to immunotherapy, radiotherapy and combinations of these modalities with chemotherapy. Accordingly, treatment with a single agent, or therapeutic modality, could rarely be expected to be curative in non-resectable human cancer. Since not all cells within a neoplasm have identical malignant potentials, with some being much more capable than others of producing metastases [6], the challenge is to devise screening methods for selecting antineoplastic regimens against the most aggressive subpopulations of a tumor, i.e. those that invade or metastasize. Integral to this approach is the concept that relevant biochemical targets of combination chemotherapy may well be located in separate cells rather than within intracellular metabolic pathways. The existence of tumor cell heterogeneity provides a new and compelling rationale for combination therapy, but one that stresses susceptibility of individual subpopulations of neoplastic cells rather than additive or synergistic interactions of agents on the indentical progeny of a homogeneous neoplasm. Accordingly, a more appropriate strategy for combination therapy schedules may be to administer chemotherapeutic or other agents sequentially, rather than concomitantly, thereby reducing additive toxicity to normal tissues.

New perspectives on the therapy of heterogeneous tumors. A major question that arises in considering tumor cell heterogeneity concerns the ability of diverse subpopulations to differ in their growth properties and still maintain intra-neoplastic diversity. Why are the slower growing cells not simply eliminated from the population by over-growth of the faster-growing subpopulations? Heterogeneity could be maintained through the appearance of new mutants in the population. Alternatively, differentiation of some cells in a population could result in phenotypically distinct cells with growth properties quite different from the parent cell. As noted previously, cultures derived from 68H tumors contain both 68H cells and morphological variants. When isolated, these variants demonstrate a variety of growth patterns; some of these grow much faster than the original 68H cells. Recently, we have found that exposure of 68H cells *in vitro* to DMF also results in the appearance of morphological variants similar to those observed after passage *in vivo*. Thus, the 68H line may provide a model for studying phenotypic instability and its contribution to tumor heterogeneity. A third mechanism for maintaining tumor heterogeneity could be control through interactions among tumor cell subpopulations. Foulds [33] has anticipated this control in his description of organoid tumor growth. We are developing methods to identify and study such interactions. Our data indicate that co-cultures of slow-growing 68H cells and fast-growing 168 cells grow at the same rate as 68H cells alone. Furthermore, we have found that repeated passages of such co-cultures maintain intact the initial proportions of the two cell lines. In these experiments, 68H cells were identified not by morphological characteristics, but by the presence of MMTV surface antigen. Thus, morphological instability does not contribute to these results. The mechanism by which 68H cells control the growth rate of 168 cells is unknown and is currently being actively investigated in our laboratory.

Effective control of neoplastic growth could depend upon our ability to manipulate tumor heterogeneity. Methods of directing and controlling the differentiation of tumor cells could potentially limit heterogeneity through the conversion of entire populations of malignant cells to a single phenotype. Moreover, if this single phenotype were to be benign, as noted after DMF treatment *in vitro*, the disease would be effectively arrested. If complete differentiation were not achieved, however, limiting tumor heterogeneity could still be therapeutically useful, since fewer cell types would represent fewer specific targets for therapy, thereby increasing the efficacy of any particular antineoplastic regimen. Furthermore, maturational therapy may produce a synchrony among the converging subpopulations, rendering them more sensitive to cycle-specific agents.

An alternative approach to dealing with the problems posed by tumor heterogeneity would be to attack the intercellular control mechanisms through which it is maintained. This could lead to a simplification of the tumor by exposing all the subpopulations to the selection pressures exerted either by the host or by therapy,

preventing any subpopulation from hiding within a protective intra-tumor sanctuary. This approach differs conceptually from that of maturational therapy, since in one case rigid control of differentiation is imposed, whereas in the other, total elimination of control is essential.

Although induction of differentiation and maintenance of heterogeneity in tumors are achieved by mechanisms that remain largely unknown, it is imperative that oncologists recognize their significance and consider their potential for influencing current antineoplastic therapy and increasing its effectiveness in the future.

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