Interactions between Tumor Subpopulations Affecting Their Sensitivity to the Antineoplastic Agents Cyclophosphamide and Methotrexate[1]

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ABSTRACT

The hypothesis that individual tumor subpopulations which differ in sensitivity to chemotherapeutic agents can influence each other's drug sensitivity was investigated using a set of mouse mammary tumor subpopulations derived from the same tumor. In one set of experiments, syngeneic mice were given injections of cyclophosphamide (CY)-sensitive line 168 cells and relatively insensitive line 410 cells on opposite flanks. Other mice received bilateral injections of only 168 or only 410 cells. CY administration was begun 2 days later and continued once a week for 4 weeks. The sensitivity of line 168 tumors was not affected by line 410 tumors, but the sensitivity of line 410 tumors was increased by the presence of line 168 tumors. The ability of line 168 tumors to increase the CY-sensitivity of line 410 tumors was not altered by irradiating (400 rads) the hosts 2 days before tumor cell injection. Mice bearing line 168 tumors only or both line 168 and line 410 tumors were more sensitive to the acute toxic effects of single, high doses of CY than were the line 410 tumor-bearing mice, suggesting that effects on CY activation are responsible for the drug sensitivity interaction.

In a second set of experiments, cells of subpopulations which differed in sensitivity to methotrexate (MTX) were cocultured in vitro in the presence or absence of MTX. In the presence of MTX-sensitive line 410.4 cells, the sensitivity of cells of lines 67 and 168 was increased. The sensitivity of cells of another relatively insensitive population, T68H clone 8, was not affected. Thus, the sensitivity of some mammary tumor subpopulations to both CY and MTX can be influenced by the presence of other subpopulations that differ, when tested alone, in sensitivity to these agents.

INTRODUCTION

The existence of subpopulations of tumor cells which differ in numerous properties has been amply documented for a number of animal and human cancers (7, 12). A major focus in such studies of tumor heterogeneity has been the demonstration of differential drug sensitivities among subpopulations from single tumors (1–3, 9–11, 14, 18–20). In these studies, sensitivity to therapeutic drugs has been assessed in individual subpopulations growing in isolation. We reported recently that the growth characteristics of individual subpopulations of a mouse mammary tumor can be influenced by the presence of other subpopulations of the same tumor (16). The purpose of the present study was to see whether interactions could also be demonstrated for sensitivity to drugs. We report here 2

examples of such interactions, one involving an increase of sensitivity to CY[3] in vivo and the other to MTX in vitro.

MATERIALS AND METHODS

Mice. Male BALB/cfC3H mice, 3 to 5 months old, were purchased from the Cancer Research Laboratory, University of California, Berkeley, Calif.

Tumor Cell Lines. The Tumor lines 410, 168, 67, and 68H were derived from the same single, spontaneously arising mammary tumor of a BALB/cfC3H mouse (6, 11). T68H clone 8 is a variant produced after one in vivo passage of line 68H (8). Line 410.4 was derived from the fourth transplant generation of the line 410 tumor (4). All lines were grown in Waymouth's medium supplemented with 7% horse serum, 7% newborn calf serum, 1% fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml).

CY Sensitivity Experiments. Cells for injection into mice were harvested from cell culture with 0.125% trypsin in 0.05% EDTA, washed once, and resuspended in 0.9% NaCl solution. Tumor cells were injected in 0.1 ml of 0.9% NaCl solution s.c. into the flanks on either side of mice, ventrally, at the level of the inguinal mammary gland. Mice were examined 2 times/week for palpable tumors. The number of tumor-free days for each animal developing a tumor was defined as the number of days between injection and the appearance of a palpable, progressively growing tumor for animals developing a tumor and was set at the termination date for the experiment for those animals not developing a tumor in that period (specified in "Results" for each experiment). The parameter "mean tumor-free days" includes data for all animals given injections, thus combining latency period and incidence into a single statistic.

In some experiments, mice were immunosuppressed 2 days prior to tumor cell injection with 400 rads whole-body γ-irradiation given at 105.9 rads/min (^{137}Cs irradiator, Model GR-6A; U. S. Nuclear Corp.).

CY. CY (Cytoxan; Mead-Johnson) was dissolved in water and diluted with 0.9% NaCl solution to a volume such that the desired dose was delivered as 0.1 ml/10 g mouse body weight. Freshly prepared solutions were injected i.p.

MTX Sensitivity Experiments. Sterile tissue culture plastic coverslips (diameter, 25 mm; Lux Thermanox) were placed in 100-mm Petri dishes. Each coverslip was stuck down with a spot of autoclaved silicone stopcock grease (Dow Corning). Freshly detached cell suspensions were placed in each Petri dish at a density of 8×10^3 cells/sq cm and allowed to attach for 24 hr. The coverslips with attached cells were transferred to 60-mm Petri dishes; 2 coverslips bearing the same or different cell lines were placed in each dish. Medium that contained varying doses of MTX (Lederle Laboratories, Pearl River, N. Y.) was immediately placed in each dish. After 48 to 72 hr, cells were removed with trypsin and counted by hemacytometer. Alternatively, coverslips were removed and placed in 35-mm dishes containing 0.1 M citric acid. After incubation for 15 to 30 min at 37°, cells were scraped off the coverslips and lysed with a drop of Zap-Isoton II (Coulter Electronics, Hialeah, Fla.), and the released nuclei were diluted and counted in a Model ZH Coulter Counter. Experiments in which a blank coverslip was incubated with a cell-containing coverslip were carried out in order to estimate the amount of cell transfer from

[3] The abbreviations used are: CY, cyclophosphamide; MTX, methotrexate.

one coverslip to the other; essentially, no cells were found on the second coverslip after 72 hr.

Statistical Analysis. The log-rank test (15) was used to test for differences between groups of mice treated with CY. Student's *t* test was used to test for differences between groups of MTX-treated cells.

RESULTS

Sensitivity of Tumors 410 and 168 to CY

We reported previously that subpopulations of a single mouse mammary tumor differed markedly in sensitivity to CY *in vivo* (11). In order to see whether subpopulations can influence each other's sensitivity to that drug, we chose a relatively sensitive subpopulation, line 168, and a relatively resistant subpopulation, line 410. Our experimental design was similar to that used to detect growth interactions between tumor subpopulations (16). Some mice were given injections on opposite sides of cells of line 168 and line 410, and other mice received line 168 cells or line 410 cells on both sides. The effect of CY was evaluated on the growth of these 2 tumors.

Influence of Line 168 Tumors on the Sensitivity of Line 410 Tumors to CY. Male BALB/cfC3H mice were given bilateral injections of cells of lines 168 and 410. Two days later and at weekly intervals for 4 weeks, CY (50 mg/kg) or 0.9% NaCl solution was injected i.p. The results of these treatments on the growth of line 410 tumors are shown on Table 1. As can be seen, the line 410 tumors growing in the NaCl solution-treated mice were unaffected by the presence of line 168 tumors. Line 410 tumors were insensitive to CY when they were growing by themselves. The combination of line 168 tumors and CY, however, resulted in an increase in the mean tumor-free days of mice bearing line 410 tumors.

CY has well-known immune modulating effects, ranging from immunosuppression (5) to overall stimulation of cell-mediated reactivity (13, 21). We have demonstrated previously a complex immune relationship among our tumor subpopulations including several instances of nonreciprocal cross-reactivities (17). In order to see whether the results shown in Table 1 were the consequence of altered immunity, the experiment was repeated in mice immunosuppressed by 400 rads administered 2 days prior to tumor cell injections. As before, CY treatment was begun 2 days after tumor cell injection but was given weekly for only 2 weeks. The results in Chart 1 show that the
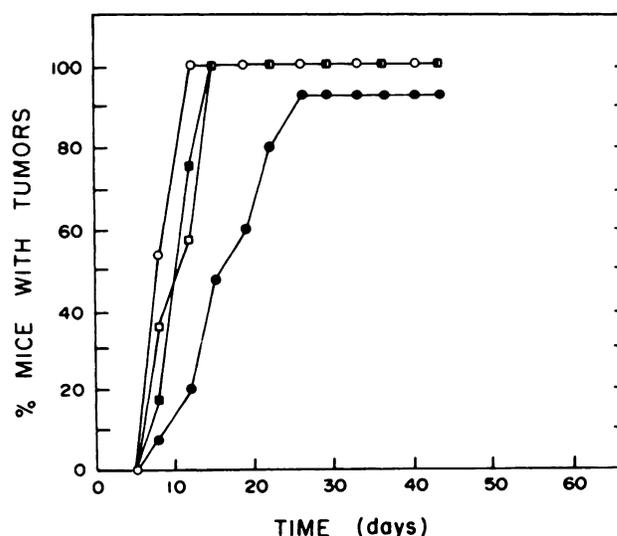

Chart 1. Sensitivity of Tumor 410 to CY in irradiated mice. Male BALB/cfC3H mice were irradiated with 400 rads 2 days prior to injection of 10^5 cells of Tumor line 410. On the same day, they were also given s.c. injections of 10^5 cells of either line 410 or line 168 on the opposite flank. Two days after tumor cell injection, either 0.9% NaCl solution or CY (35 mg/kg) was administered i.p. and repeated 1 week later. The incidence of Tumor 410 is shown: NaCl solution treatment, Tumor 410 opposite (□, 14 tumors); NaCl solution treatment, Tumor 168 opposite (○, 12 tumors); CY treatment, Tumor 410 opposite (■, 12 tumors); CY treatment, 168 opposite (●, 15 tumors). The probability that differences between groups are due to chance as determined by log rank test: $p < 0.0001$ for all groups; $p = 0.0009$ for 2 CY-treated groups.

influence of 168 tumors on line 410 sensitivity to CY is not dependent on an intact immune system. Once again, line 410 tumors were sensitive to CY only in the presence of 168 tumor.

Influence of 410 Tumors on the Sensitivity of Line 168 Tumors to CY. We have reported that line 410 tumors can retard the growth of line 168 tumors injected on the opposite side of syngeneic mice (16). This "growth interaction" appears to be due to the induction of an immune response by 410 tumors against the nonimmunogenic, but immunosensitive, line 168 tumors. It was of interest to see whether the sensitivity of 168 tumors to CY would be influenced by 410 tumors. In 2 separate experiments, one of which is shown in Table 2, the CY sensitivity of 168 tumors was not affected by the presence of 410 tumors. Similarly, the presence of the CY-sensitive Tumor 410.4 did not change the sensitivity of Tumor 168 to CY (not shown). The results of the experiment in Table 2 also demonstrate that the ability of 410 tumors to inhibit growth of 168 tumors was not affected by CY. The ratio between the mean tumor-free days of 168 tumors growing in the presence of Tumor 410 and those of 168 tumors growing alone was not significantly affected by CY.

Influence of 410 Tumors and 168 Tumors on the Toxicity of CY. CY is activated *in vivo* by the cytochrome P-450 system (5). One possible mechanism by which Tumor 168 would affect Tumor 410 sensitivity is through an effect on drug activation. If this was true, one would expect that the toxicity of CY would also be influenced by the presence of Tumors 168 and 410. BALB/cfC3H mice were given bilateral injections of line 168 and 410 cells, line 168 cells, or line 410 cells. After tumors had reached an average size of 20 sq mm, the mice were given injections of CY ranging from 200 to 500 mg/kg. Mice bearing line 168 tumors were more sensitive to the acute toxic effects of CY than were mice bearing only line 410 tumors. This was

Table 1
Sensitivity of Tumor 410 to CY in mice bearing Tumor 168

| | | | Growth of 410 tumors | |
Group	Tumor on opposite flank[a]	Treatment[b]	Incidence	Mean tumor-free days[c]
1	410	0.9% NaCl solution	19/20 (95)[d]	16
2	168	0.9% NaCl solution	10/11 (91)	17
3	410	CY (50 mg/kg)	18/20 (90)	19
4	168	CY (50 mg/kg)	7/10 (70)	32[e]

[a] Male BALB/cfC3H mice were given s.c. injections of 10^5 cells of tumor line 410. On the same day, they were also given s.c. injections of 10^5 cells of either line 410 or line 168 on the opposite flank.

[b] Treatment was initiated 2 days after tumor cell injections and given i.p. once a week for 4 weeks.

[c] Computed as described in "Materials and Methods," using 60 days as the experiment termination date.

[d] Numbers in parentheses, percentages.

[e] Probability that differences between groups are due to chance as determined by log rank test: 4 *versus* pooled 1 to 3, $p < 0.05$.

Table 2

Sensitivity of Tumor 168 to CY in mice bearing Tumor 410

Tumor on opposite flank[a]	Treatment[b]	Growth of Tumor 168		MTFD[d] (line 410 opposite)
		Incidence	MTFD[c]	MTFD (line 168 opposite)
168	0.9% NaCl solution	19/20 (95)[e]	28	
410	0.9% NaCl solution	9/10 (90)	37	1.3
168	CY (35 mg/kg)	17/18 (94)	40	
410	CY (35 mg/kg)	6/10 (60)	61	1.5
168	CY (50 mg/kg)	14/20 (70)	53	
410	CY (50 mg/kg)	6/12 (50)	67	1.3

[a] Male BALB/cfC3H mice were given s.c. injections of 10^5 cells of tumor line 168. On the same day, they were also given s.c. injections of 10^5 cells of either line 168 or line 410 on the opposite flank.

[b] Treatment was initiated 2 days after tumor cell injections and given i.p. once a week for 4 weeks.

[c] Computed as described in "Materials and Methods," using 80 days as the experiment termination date. MTFD, mean tumor-free days.

[d] MTFD, from previous column.
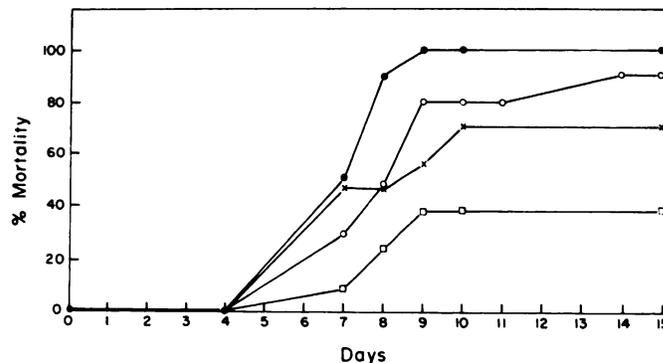
[e] Numbers in parentheses, percentages.

Chart 2. Toxicity of CY in tumor-bearing mice. Male BALB/cfC3H mice were given injections of Tumor 410 cells on both sides (□, 8 animals), Tumor 168 cells on both sides (●, 8 animals), or Tumor 410 cells on one side and Tumor 168 cells opposite (○, 16 animals). Some mice were not given injections of tumor cells (×, 7 animals). Mice were given i.p. injections of CY (350 mg/kg) when tumors reached an average size of 20 sq mm. The probability that differences between groups are due to chance as determined by log rank test: $p = 0.012$ for all groups; $p = 0.005$ for group bearing line 168 tumors only *versus* group bearing line 410 tumors only; $p = 0.034$ for group bearing line 410 tumors only *versus* group bearing line 410 and line 168 tumors.

Table 3

Effect of line 410.4 on the MTX sensitivity of other cell lines

Experiment	Cell line	ID_{50} (nM)[a]	ID_{50} in presence of line 410.4 (nM)[b]
1	67	15	7
2	67	17	6
3	67	22	7
4	67	40	23
5	67	28	10
6	67	14	11
7	168	18	7
8	T68H clone 8	22	22

[a] ID_{50} concentration of MTX at which the increase in cell number after 72 hr was only one-half as much as the increase in the absence of MTX. The ID_{50} values were derived from plots, like those shown in Chart 3, of the logarithm of MTX concentration *versus* cell number. MTX concentrations between 4 and 64 nM were used in all experiments. Cells were grown on coverslips in the presence of other coverslips plated with the same cell type.

[b] Cells were grown on coverslips in the presence of other coverslips plated with line 410.4.

most clearly seen at the 350-mg/kg dose for which the data are presented in Chart 2. Host toxicity paralleled tumor sensitivity in that line 168 tumors were more sensitive to CY and mice bearing line 168 tumors were more sensitive to toxic doses of CY than were line 410 tumors and line 410 tumor-bearing hosts, respectively. Line 410 tumors were more sensitive to CY in the presence of line 168 tumors than in the presence of a second line 410 tumor, and hosts bearing both line 410 plus line 168 tumors were more sensitive to CY toxicity than were hosts bearing only line 410 tumors.

Sensitivity of 410.4 and Other Cell Subpopulations to MTX

Another drug to which mammary tumor subpopulations display a range of sensitivity is MTX. We investigated the effect of subpopulation interactions to MTX using an *in vitro* approach. Cells of subpopulations chosen for differences in MTX sensitivity were plated on coverslips, and 2 coverslips bearing the same or different subpopulations were placed together in Petri dishes. Cell growth was monitored in the presence of various MTX concentrations.

Table 3 summarizes 6 experiments in which line 410.4 cells increased the sensitivity of line 67 to MTX (as indicated by a decrease in the concentration of MTX required to reduce tumor cell replication by 50%); sensitivity of other tumors may or may not be affected by the presence of line 410.4 cells (Table 3, Experiments 7 and 8). Thus, the presence of line 410.4 cells enhanced the MTX sensitivity of both line 67 and line 168 cells, but the MTX sensitivity of T68H clone 8 cells was not affected. In no case was line 410.4 sensitivity altered by the presence of other cell types (not shown). Chart 3 presents data from one such experiment in detail. Line 410.4 cells, the more MTX-sensitive line, were equally affected by MTX whether in the presence of less sensitive line 67 cells or in the presence of a second coverslip bearing 410.4 cells. Line 67 cells, however, were more sensitive to MTX in the presence of line 410.4 cells. In most experiments, cell viabilities were measured by trypan blue exclusion. Cell viability, even at the highest MTX concentrations, remained above 90% in all groups. This suggests that the data on Table 3 reflect changes in the rate of cell replication rather than cytotoxicity.
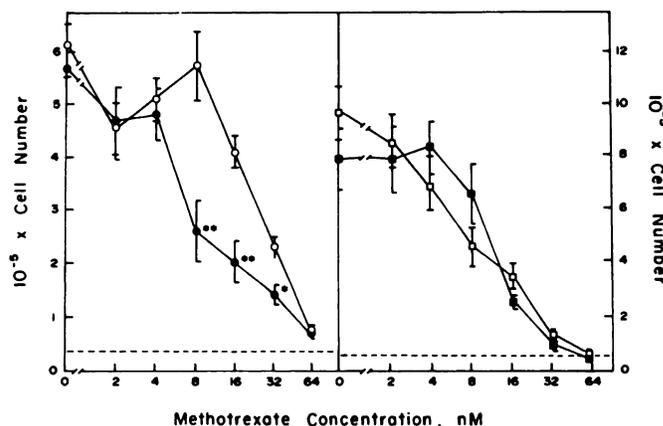

Chart 3. Transfer of MTX sensitivity *in vitro*. Left, sensitivity of line 67 cells. Coverslips bearing line 67 cells were placed in 60-mm dishes in the presence of other coverslips bearing either line 67 cells (○) or line 410.4 cells (●). Medium contained MTX concentrations as shown. After 72 hr, line 67 cells were counted by hemacytometer. - - - -, cell number at the start of the experiment. *Points*, mean of 6 replicates; *bars*, S.E. At each MTX concentration, the cell numbers in the 2 groups were compared by Student's *t* test. ☆, $p < 0.05$; ☆☆, $p < 0.01$. *Right*, sensitivity of line 410.4 cells in the same experiment. The line 410.4 cell number is shown. The other coverslip contained line 67 cells (□) or line 410.4 cells (■).

DISCUSSION

We and others have shown that individual subpopulations of single neoplasms can be differentially affected by cytotoxic therapy (1–3, 9–11, 14, 18–20). A natural conclusion from this demonstration is the idea that the characteristic drug sensitivities demonstrated by the isolated subpopulations will continue to be expressed in the mixed-population milieu of a whole tumor. The results presented in this report suggest that the situation is more complex. The sensitivity of some subpopulations to 2 agents, CY and MTX, have been found to be influenced by the presence of other subpopulations. This has been demonstrated both *in vitro* (MTX) and *in vivo* (CY). With both drugs, the direction of the interaction has been toward increasing the sensitivity of the less sensitive cell subpopulation. It is probable that, with other drugs or other subpopulations, other types of interactions also exist.

The mechanisms of the interactions demonstrated here are not yet clear. Given the different modes of action of the 2 drugs and the different circumstances in the *in vivo versus in vitro* methods used, it is improbable that the same mechanisms are responsible in the CY and the MTX experiments. At this time, we favor the hypothesis that the CY results are due to differences in the kinetics or extent of drug activation in mice bearing the 2 different tumor types.

We are currently investigating several possibilities for the mechanism of the MTX interaction. It is not probable that alteration of MTX transport is the target of the interaction, since line 410.4 cells and line 67 cells have very similar abilities to transport that drug (data not shown). Other potential mechanisms include changes in the level of dihydrofolate reductase or alterations in endogenous purine or pyrimidine pools or folate levels (5). The simple idea that MTX-treated line 410.4 cells release some nonspecific "factor" inhibitory to the growth of other cells in general seems belied by the observation that not all "other" subpopulations are affected [for example, T68H clone 8 cells (Table 3)]. The hypothesis that MTX-treated line 410.4 cells use some essential nutrient is also improbable in view of the results of preliminary experiments (not shown) which indicate that conditioned medium from MTX-treated line 410.4 cells is no more inhibitory to line 67 cells than is conditioned medium from MTX-treated line 67 cells or unconditioned medium incubated for a similar time with MTX prior to testing. Because the coverslip protocol used to demonstrate the transfer of MTX sensitivity prevents cell contact, the failure of line 410.4-conditioned medium to mimic line 410.4 cells on coverslips suggests that, whatever the target of the interaction, it involves the transfer of a labile mediator.

Regardless of the underlying biochemical explanation for our observations, we believe that the demonstration of drug sensitivity interactions among tumor cell subpopulations adds a new dimension to understanding the role of heterogeneity in tumor therapy. For example, it seems possible that the ability of one subpopulation to affect the drug sensitivity of another subpopulation may be involved in certain instances of drug synergisms in which increased sensitivity to a second drug is involved. Furthermore, it may be that interactions such as the ones demonstrated here could mask the presence of biochemically resistant tumor subpopulations by allowing only their emergence after the "controlling" subpopulation has been eliminated. If this is so, it may be necessary to revise our concepts of the factors governing the origin and emergence of drug-resistant tumor cells. On the other hand, the knowledge of the mechanisms by which tumor cells can influence each other's drug sensitivity may suggest new approaches, *i.e.* different drug combinations and sequence scheduling, that would enhance therapeutic efficacy. Appreciation of drug sensitivity interactions may also lead to modifications in current approaches to assessing the drugs most likely to be effective against individual patient's cancers.

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