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A Dominated and Resistant Subpopulation Causes Regrowth after Response to 1,3-Bis(2-chloroethyl)-1-nitrosourea Treatment of a Heterogeneous Small Cell Lung Cancer Xenograft in Nude Mice[1]

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ABSTRACT

In order to address the question of the influence of a primarily chemoresistant tumor cell subpopulation on the progression of a heterogeneous tumor after cytotoxic therapy, *in vitro* established human small cell lung cancer cell lines of a 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)-sensitive (592) and a resistant (NYH) tumor were used to produce mixed solid tumors in nude mice. Mixtures of 592/NYH (9:1 and 1:1) were inoculated s.c. After 3–4 weeks of tumor growth, the mice were stratified according to tumor size and randomized to treatment with BCNU 40 mg/kg i.p. (10% of lethal dose) or no treatment. Tumor growth curves were used to calculate the effect of the treatment, and changes in the relative proportions of 592 and NYH in the mixed tumors were monitored by flow cytometric DNA analysis by which the two cell lines were distinguishable due to differences in DNA content. A significant response was demonstrated in the 9:1 mixed tumors in which only 592 cells were detectable at the start of the treatment. The response was short and less pronounced compared with tumors containing only 592. In the regrowing tumors after treatment, only NYH was detected. In untreated 9:1 mixed control tumors, only 592 cells were detectable throughout the entire observation period. It is substantiated that the 592 cells were able to inhibit the the growth of the NYH cells completely when grown together in 9:1 mixed tumors. This was not the case in the 1:1 mixed tumors. The 1:1 mixed tumors did not respond to BCNU, although 592 was eradicated. These results indicate that resistant and undetectable (dominated) subpopulations in heterogeneous tumors may be responsible for relapse and that the fractional size and the growth characteristics of the resistant subpopulation may determine the magnitude of the clinical response to cytotoxic treatment.

INTRODUCTION

It has become evident and generally accepted that solid tumors including SCLC[3] are cellularly heterogeneous (1–7). This heterogeneity involves many phenotypic characteristics, one of which may be differences among the subpopulations in sensitivity to cytotoxic drugs (8). Intratumoral cellular diversity is probably the result of genetic instability of the tumor cells leading to emergence of new subpopulations with diverse phenotypic characteristics (9). According to the Goldie-Coldman hypothesis (10), spontaneous mutations lead to resistant subpopulations already at the time of diagnosis, depending on the size of the tumor cell population and the mutation rate.

Over the past few years, the phenomenon of clonal interaction between subpopulations has been demonstrated *in vivo* in several murine and human tumor systems (11–21). We have shown interaction between two subpopulations of a single human SCLC xenografted into nude mice.[4] One of the clones was able to dominate the other when grown in close cellular contact in mixed solid tumors.

In clinical oncology, many malignant tumors are primarily resistant or only slightly sensitive to cytotoxic treatment. Most sensitive tumors like SCLC develop resistance during the course of the treatment, or if a complete remission is achieved, relapse of resistant tumor cells is commonly seen.

In order to address the question of the influence of a primarily chemoresistant tumor cell subpopulation on the evolution of a heterogeneous tumor after cytotoxic therapy, we have investigated the effect of chemotherapy (BCNU) on a mixed chemosensitive (592) and chemoresistant (NYH) human small cell lung cancer xenografted into nude mice. Since the cell lines were distinguishable by differences in cellular DNA content, the changes in the proportions of the two cell lines were monitored by flow cytometric DNA analysis of fine-needle tumor aspirates.

MATERIALS AND METHODS

Experimental Design. The *in vivo* sensitivity to BCNU (Carmustine) 40 mg/kg i.p. (10% of letahl dose) of the two human SCLC cell lines 592 and NYH was tested. Mixed solid tumors were established in nude mice by s.c. inoculation of 9:1 and 1:1 mixtures of 592/NYH, and the tumor-bearing animals were treated with BCNU 40 mg/kg i.p. Untreated mixed tumors served as controls. The effect of BCNU was evaluated by tumor growth curves, and the changes in the relative proportions of 592 and NYH in the mixed tumors were monitored by repeated flow cytometric DNA analysis performed on fine-needle aspirations from the tumors.

Cell Lines. The human SCLC cell lines used were NCI-N592 (592; Ref. 22) and OC-NYH (NYH; Ref. 23). The cell lines differed in sensitivity to BCNU *in vitro* (24).

Mice. Six-week-old athymic nude male NMRI mice (Bomholtgaard Breeding and Research Centre, Ltd., Ry, Denmark) were used as tumor-bearing hosts. The animals were kept under sterile conditions in laminar air flow benches and allowed sterilized food and water *ad libitum*.

Cell Suspensions. The cell lines were maintained at 37°C in RPMI 1640 with 10% fetal calf serum in a humidified atmosphere with 7.5% CO_2. At regular intervals, the cell lines were reestablished from frozen subcultures in order to reduce or avoid changes in sensitivity and other cellular characteristics (genetic drifting). The cell lines were free of contamination by *Mycoplasma*. DNA indices as measured by DNA flow cytometry (see below) were 1.45 and 1.30 for 592 and NYH, respectively. Five $\times 10^6$ cells (0.1 ml) of either 592 or NYH were inoculated s.c., and after 3–4 weeks of tumor growth, the tumors were tested for sensitivity to BCNU. Mixed tumors were produced by s.c. inoculation of 9:1 and 1:1 mixtures of 592/NYH cells, each inoculum of 0.2 ml containing 5×10^6 cells of 592 and 5×10^5 cells of NYH or 5×10^6 cells of 592 and 5×10^6 cells of NYH, respectively.

Tumor Growth Curves. The mice were observed three times weekly for tumor take. After tumor take, the tumors were measured bidimensionally with callipers three times weekly. The product of the measurements (mm^2) served to construct the growth curves. Using a computer program (25), the mean TD

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[3] The abbreviations used are: SCLC, small cell lung cancer; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; TD, tumor volume doubling time; GD, growth delay; SGD, specific growth delay; FCM, flow cytometry.

[4] K. Aabo, L. L. Vindeløv, and M. Spang-Thomsen. Clonal dominance between subpopulations of mixed small cell lung cancer xenografts in nude mice, submitted for publication.

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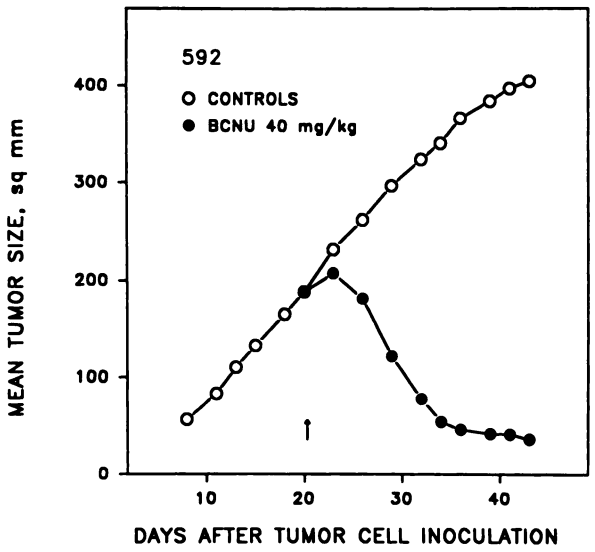

Fig. 1. Mean tumor growth curves of the human SCLC xenograft 592 in nude mice before and after treatment (*arrow*) with 40 mg/kg BCNU i.p. (24 tumors) and of untreated control tumors (22 tumors).

at a preselected tumor volume of 350 mm^3 was calculated. The effect of the treatment was determined in terms of growth delay, GD = time to grow to twice the tumor volume at the time of treatment, and specific growth delay, SGD = (TGD-CGD)/CGD, where TGD and CGD are the calculated growth delays of the treated and the untreated tumors, respectively (25).

Treatment. After 3–4 weeks of tumor growth, the animals were stratified according to tumor size and randomized to treatment with BCNU or no treatment. BCNU (Carmustine; Bristol) was dissolved (100 mg of BCNU plus 3 ml of absolute alcohol plus 97 ml of sterile water) and injected i.p. in a dose of 40 mg/kg (10% lethal dose). Control animals were injected with solvent alone.

FCM DNA Analysis. The cell mixtures and the fine-needle aspirations from the mixed solid tumors before and after treatment were analysed by FCM using a FACS III (Becton Dickinson, Sunnyvale, California). The proportion of each cell line was estimated by a maximum likelihood method (26). The detection limit was approximately 5%. The DNA index of the tumor cells was determined as the ratio of the DNA content of the tumor G_0+G_1 cells to that of human diploid cells by the use of chicken and trout RBCs as internal standards (27).

RESULTS

The mean growth curves of 592 and NYH tumors and their response to 40 mg/kg BCNU are shown in Figs. 1 and 2. The 592 tumors responded well to treatment. Two tumors disappeared, and regrowth of the remaining tumors was so delayed that GD and SGD could not be calculated for the applied observation period of 90 days. Four treated animals in the 592 group (seven tumors) were still alive when the experiment was terminated. BCNU had no effect on the NYH tumors, which had a longer lag period before tumor take followed by a faster growth compared with 592. Thus, the mean TD for untreated 592 was 9.5 days and for untreated NYH 3.0 days (Table 1). The cell cycle distributions and the DNA indices of untreated tumors are given in Table 2. More 592 cells were in G_0+G_1 compared with NYH which had more cells in S, corresponding to the faster growth observed in the growth curves. The treated 592 tumors showed accumulation of cells in G_2 +M, whereas the treated NYH tumors showed no changes in the cell cycle distributions (data not shown).

A significant tumor response was demonstrated in the 9:1 mixed 592/NYH tumors (Fig. 3). The response was not as pronounced as in unmixed 592 tumors, and the response duration was short. During regrowth of the treated tumors, the growth curve was steeper with a TD resembling that of unmixed NYH tumors. The untreated 9:1 mixed control tumors grew phenotypically as unmixed 592 tumors. Thus, the TD of the 9:1 mixed control tumors was 7.0 days compared with 9.5 days for unmixed 592 tumors. During regrowth, the TD was 2.3 days compared with 3.0 days for unmixed NYH tumors (Table 1). The calculated BCNU-induced GD in the 9:1 mixed tumors was 11.2 days, corresponding to a SGD of 1.25 (Table 1). The relative proportions of 592 and of NYH in the intended 9:1 inoculate was 90 and 10%, respectively, as determined by FCM (Fig. 4). In all tumors, only 592 cells were detectable at the start of the treatment 3 weeks after tumor cell inoculation (Fig. 4). During regrowth of the tumors 3 weeks after the treatment, only NYH could be detected in the fine-needle tumor

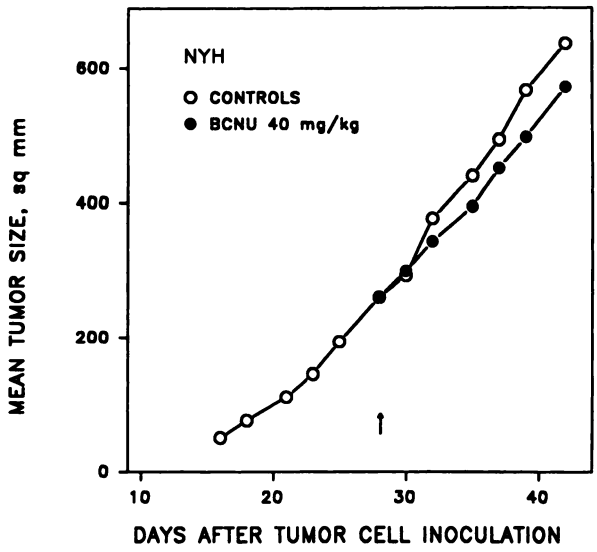

Fig. 2. Mean tumor growth curves of the human SCLC xenograft NYH in nude mice before and after treatment (*arrow*) with 40 mg/kg BCNU i.p. (15 tumors) and of untreated control tumors (16 tumors).

Table 1 *Growth parameters of two human SCLC xenografts 592 and NYH and of mixed tumors, treated and untreated with 40 mg/kg BCNU i.p.*

Tumor	No. of tumors	Mean TD (days)	Mean GD (days)	SGD
592				
Controls	22	9.5	10.8	0
Treated	24	–[a]	–	–
NYH				
Controls	16	3.0	5.7	0
Treated	15	3.8	7.0	0.23
592/NYH (9:1)				
Controls	14	7.0	8.9	0
Treated	13	2.3	20.1	1.25
592/NYH (1:1)				
Controls	9	2.6	5.9	0
Treated	9	3.4	7.6	0.30

[a] Due to the pronounced effect of BCNU, TD, GD, and SGD could not be estimated since regrowth was insufficient (the tumors did not reach the double tumor volume) during the observation period.

Table 2 *DNA indices and cell cycle distributions of two human SCLC xenografts 592 and NYH as measured by FCM DNA analysis on fine-needle tumor aspirates 3–4 weeks after s.c. tumor cell inoculation in nude mice (17 tumors in each group).*

		Median cell cycle distributions in percentages (range)		
Cell line	DNA index	$G_0 + G_1$	S	G_2 + M
592	1.45	56 (46–64)	34 (26–45)	10 (6–15)
NYH	1.30	46 (32–51)	49 (40–59)	5 (2–14)

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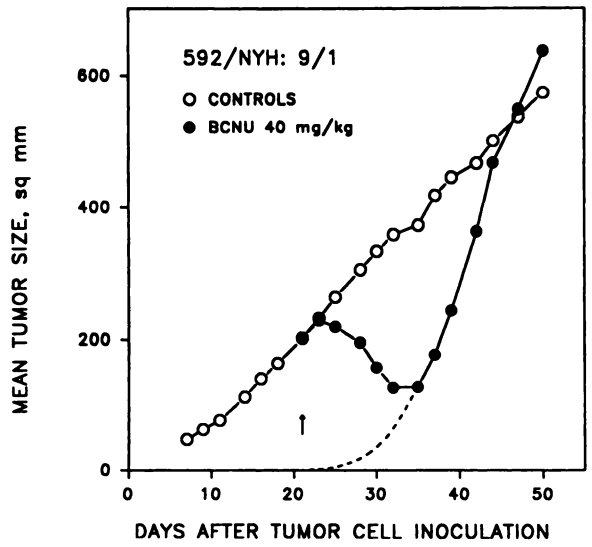

Fig. 3. Mean tumor growth curves of 9:1 mixed BCNU-sensitive (592) and BCNU-resistant (NYH) human SCLC xenografts before and after treatment (*arrow*) with 40 mg/kg BCNU i.p. (13 tumors) and of untreated mixed control tumors (14 tumors). The *stippled line* is a backward extrapolation of the NYH regrowth curve estimated from a transformed Gompertz function (see "Discussion").

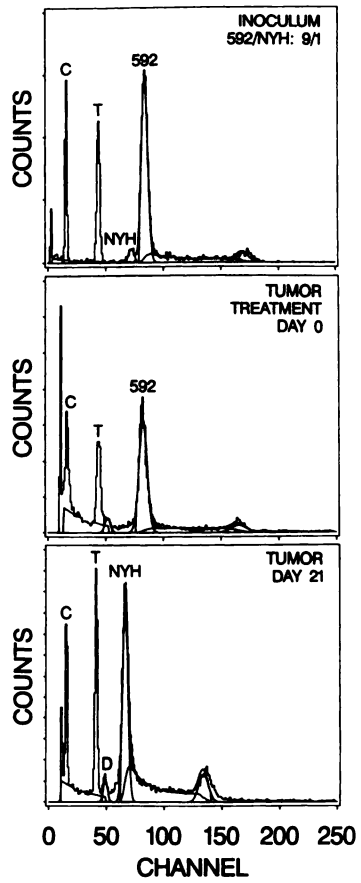

Fig. 4. Series of FCM DNA histograms showing the development in composition of an intended 9:1 mixed 592/NYH SCLC xenograft in nude mice. The FCM-determined proportions of 592 and of NYH cells in the inoculum were 90 and 10%, respectively. At the time of treatment 3 weeks after tumor cell inoculation, only 592 could be detected in the fine-needle tumor aspirates. At tumor progression 21 days after the treatment, only the BCNU-resistant and previously undetectable (dominated) NYH subpopulation was detectable. In the untreated mixed control tumors, NYH was undetectable throughout the observation period (data not shown). *C*, chicken RBCs; *T*, trout RBCs; *D*, diploid host cells.

aspirates (Fig. 4). The NYH cells appeared in the first FCM histogram after 2 weeks. In the mixed control tumors, only 592 cells were detectable throughout the entire observation period.

In the experiment in which the cells were mixed in an intended 1:1 proportion, the growth of the mixed tumors were similar to the growth of unmixed NYH tumors (Fig. 5). No response to BCNU treatment could be detected (Fig. 5). Thus, the mean TD was 3.4 days for the BCNU-treated tumors and 2.5 days for the untreated control tumors, and the calculated SGD was 0.30 (Table 1). The proportions of 592 and NYH cells in the inoculum were 64 and 36%, respectively (Fig. 6). Four weeks later at the start of the BCNU treatment, the proportions of 592 and NYH cells in the tumor representing the median were 47% (range, 0–56%) and 53% (range, 44–100%), respectively (Fig. 6). Nine days after the treatment, all tumors contained only NYH cells, indicating that the 592 cells had been eradicated by the BCNU treatment (Fig. 6).

DISCUSSION

The experiments presented in this communication demonstrated that the BCNU-sensitive SCLC cell line 592 was able to dominate the faster growing and BCNU-resistant SCLC line NYH but only when grown in excess in mixed solid tumors (592/NYH; 9:1) in nude mice. This was not the case if the cells were growing in 1:1 mixed xenografts, indicating that the dominance phenomenon in this case was not absolute, as shown previously with subpopulations of both murine (13, 21) and human tumors.[4]

Using a transformed Gompertz function (28), the regrowth curve of the 9:1 mixed treated tumors containing only NYH cells (Fig. 3) was extrapolated backward to the time of the BCNU treatment (25). A tumor area of 0.33 mm^2 was calculated corresponding to a volume of 0.067 mm^3 and provided that the tumor tissue contained 5×10^8 viable cells per cm^3; this means that the tumors regrew from approximately 3.4×10^4 NYH cells. A total of 5×10^5 viable NYH cells were included in the original 9:1 mixed 592/NYH inoculum, and it is likely that only about 10% of the cells survived the initial period after inoculation until a sufficient supportive tumor microenvironment (angiogenesis) was established. Thus, the 592 cells probably completely suppressed the proliferation of the NYH cell population of the 9:1 mixed tumors.

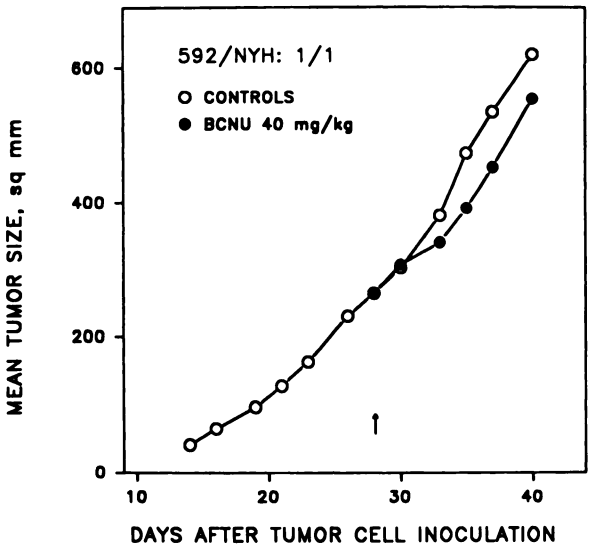

Fig. 5. Mean tumor growth curves of intended 1:1 mixed BCNU-sensitive (592) and BCNU-resistant (NYH) human SCLC xenografts before and after treatment (*arrow*) with 40 mg/kg BCNU i.p. (9 tumors) and of untreated mixed control tumors (9 tumors).

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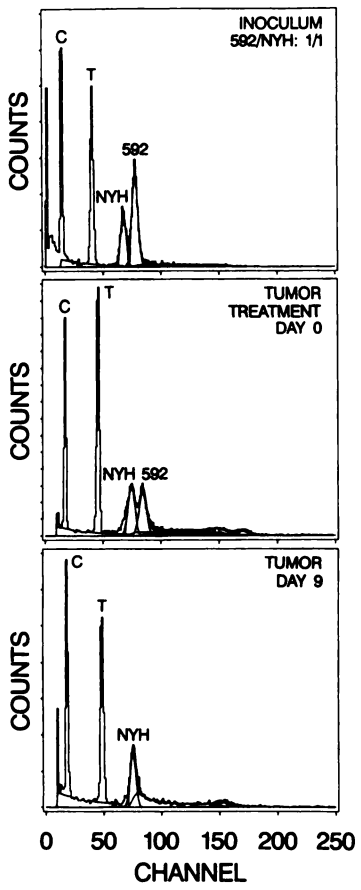

Fig. 6. Series of FCM DNA histograms showing the development in composition of an intended 1:1 mixed 592/NYH SCLC xenograft in nude mice. The FCM-determined proportions of the 592 and of the NYH cells in the inoculum were 64 and 36%, respectively. At the time of treatment 4 weeks after tumor cell inoculation, the proportions of 592 and of NYH were in median 47 and 53%, respectively. Nine days after the treatment, only NYH cells were detected in the fine-needle tumor aspirates, indicating that the 592 subpopulation had been eradicated by the BCNU treatment. *C*, chicken RBCs; *T*, trout RBCs.

After treatment of the 9:1 mixed tumors with BCNU, the response was less pronounced and of much shorter duration compared with unmixed 592 tumors. The tumor progression after the response to the treatment of the 9:1 mixed tumors was due to the dominated (nondetectable) and BCNU-resistant NYH subpopulation. In the untreated mixed control tumors, the 592 subpopulation continued to dominate the NYH population throughout the entire observation period. It is likely that the dominated and undetectable NYH subpopulation either may have acquired optimized growth conditions after eradication of the dominating 592 subpopulation or, alternatively, an inhibiting action imposed by the 592 cells on the NYH cells may have disappeared after the killing of the 592 cells. The very rapid emergence of resistant cells seen after response to chemotherapy during clinical cancer treatment may be explained by the ready growth in a primed environment of a suppressed and resistant subpopulation with faster inherent growth kinetics than a sensitive and dominating subpopulation. Thus, the development of *in vivo* resistance to cytotoxic treatment may not always be induced by the treatment itself, as seen under *in vitro* conditions using very high drug concentrations, but may be explained by growth promotion of primarily resistant and suppressed subpopulations after eradication of the sensitive and dominating population. The phenomenon of clonal dominance supports the hypothesis of Goldie and Coldman (10) according to which relapse of resistant tumor cells after response to chemotherapy may be due to resistant subpopulations present in the tumor already at the time of initiation of the treatment. This stresses the necessity of using combination chemotherapy in order to kill all subpopulations in malignant tumors.

The existence of very small, resistant and suppressed tumor subpopulations in heterogeneous tumors may constitute the major limitation to the predictivity of *in vitro* sensitivity assays.

When 1:1 mixed tumors were treated, no significant response was found (SGD, 0.30) in spite of the disappearance of the 592 subpopulation. Thus, in both the 9:1 and the 1:1 mixed tumors, the primarily chemoresistant subpopulation (NYH) was responsible for the tumor progression. As no dominance was seen in the 1:1 mixed tumors, the point at which the 592 population ceased to suppress the growth of the NYH population may lie between 50 and 90% of 592 cells.

We are aware of only two previous and recent studies which are dealing with chemotherapy of artificially heterogeneous tumors. Leith *et al.* (29) reported on an artificially mixed human colon carcinoma xenograft in nude mice. One of the subpopulations were more sensitive to mitomycin C than another subpopulation. Tumor disaggregation was followed by cloning *in vitro*. The cell lines were distinguishable by differences in colony morphology. In this study, the heterogeneous tumors reached a new stable cellular composition approximately 30 days after treatment with a higher percentage of less sensitive cells in the mixed tumors. This was in accordance with the higher killing effect of the treatment on the most sensitive subpopulation. The cell lines had similar growth characteristics. In this system, no dominating interaction between the two subpopulations seemed to influence the cellular composition of the tumors after chemotherapy.

In a mouse mammary tumor system, Miller *et al.* (30) mixed subpopulations of a melphalan-sensitive and a relatively resistant tumor and tested the effect of melphalan *in vivo* using the colony-forming ability of the tumor cells *in vitro* after tumor disaggregation. The cell lines were distinguishable in their ability to grow in selective media. In this study, the less sensitive cell line became more sensitive when grown together with the more sensitive cell line in solid tumors. This is in contrast to the findings in our study in which we found an early regrowth of the resistant subpopulation after eradication of the sensitive subpopulation by chemotherapy.

Many human malignancies are not or only a little sensitive to cytotoxic treatment, fewer are moderately sensitive, and only a few are highly sensitive. This spectrum of sensitivity may at least in part be explained on the basis of the fractional size of the primarily resistant subpopulation(s) in heterogeneous tumors. Artificially mixed tumors containing 10% of a chemoresistant SCLC subpopulation and 90% of a sensitive subpopulation showed growth characteristics similar to unmixed chemosensitive tumors. Treatment of these heterogeneous tumors resulted in a significant tumor response; however, the response was much less pronounced compared with tumors containing only the drug-sensitive cells. If one-half of the tumor consisted of the sensitive subpopulation, no tumor response was observed. Thus, the magnitude of the response may depend on the size of the sensitive population, and the duration of the remission may be a reflection of the growth kinetics of primarily resistant and dominated subpopulations. In our system, the faster growth of the NYH subpopulation resulted in a higher progression rate and thereby a reduced SGD ("poorer prognosis") despite the response of the 9:1 mixed tumors (crossing of the growth curves in Fig. 3).

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