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Variability of tumor response to chemotherapy II. Contribution of tumor heterogeneity

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Summary. The role of tumor-to-tumor variability in response to chemotherapy was investigated in mice bearing mammary adenocarcinoma 16/C treated with melphalan. Lissamine green, a triphenylmethane dye, was given systemically to delineate areas of perfusion in the tumors. The regions of low perfusion ranged from $<10\%$ to $>90\%$ of the mass of individual tumors. The variation in perfusion was as large between bilateral tumors in a mouse as between tumors in different hosts. The presence of viable cells capable of continued growth in the regions of low perfusion was demonstrated by bioassay. Concentrations of melphalan following i.p. administration varied by as much as tenfold or more between regions of low and high perfusion. Concentrations of melphalan in the well-perfused regions were similar to plasma concentrations at 30 min after administration, but elimination from the plasma was more rapid. The levels of melphalan in the tumor were higher following the initial dose than following succeeding doses in a multiple dose schedule. The results indicate that tumor-to-tumor variations in perfusion and drug distribution are major factors in variable tumor response.

Introduction

The diversity of response of histologically similar human tumors to radiation or to chemotherapy has been a challenging problem in treatment of cancer. Investigation of the problem was hindered by the belief that experimental tumors, typically derived from a single tumor and implanted in mice from inbred stock, lacked the diversity of human tumors. However, detailed evaluation of data obtained with experimental tumors indicated that variability of response to radiation and chemotherapy was present.

The variable response of experimental tumors to radiation was recognized early, and the possible role of oxygen in that response has been of interest to radiation biologists for more than 50 years. Even though an extensive literature accumulated on hypoxic fractions in experimental tumors, a definitive study was not available until Rockwell et al. [12] addressed the question of tumor-to-tumor variability of hypoxic fractions in "individual tumors from the same tumor line, implanted into the same site, in similar hosts and studied at the same size using similar experimental techniques"; these authors have reported extensive tumor-to-tumor variability in the hypoxic fractions.

The diversity of response of individual experimental tumors to chemotherapy was less apparent, probably due to the tendency of investigators to use many animals in each group and to report results as mean or median values. Tumor-to-tumor differences were clearly shown when Suit et al. [19] reported the response of individual isotransplants of a fibrosarcoma in C3H/Sed mice treated with *Corynebacterium parvum*. Fisher and Saffer [3] have attributed the heterogeneity of response of transplanted mammary tumors in C3HeB/FeJ female mice treated with a combination of *C. parvum* and cyclophosphamide to differences in tumor characteristics. Schabel et al. [17] have reported similar diversity of response of individual advanced (≥ 100 mg) solid tumors from at least nine murine tumor lines treated with effective chemotherapeutic agents. They attributed this diversity to differences in drug activation, degradation, excretion, etc., in the individual host. Nelson et al. [10] have used bilateral implants of Ridgway osteogenic sarcoma implanted in AKR male mice to evaluate the response to maximally tolerated doses of vincristine or melphalan (L-PAM) and evaluated a variety of quantal or graded parameters (tumor measurements, cures, weight loss, duration of responses, etc.). Tumor response was reported to be more closely related to host than to tumor differences. Heppner and Miller [7] have attributed the variability of response to the presence of different subpopulations within the tumor.

We have demonstrated the variable response of mammary adenocarcinoma 16/C (mam ad 16/C) tumor in B6C3F1 mice to treatment with L-PAM and studied the role of host heterogeneity in the pharmacokinetics of the drug [18]. The results indicated that host-to-host differences were not responsible for the variable tumor response when treatment was begun before tumors weighed 1.0 g. We further investigated tumor-to-tumor variability in the same tumor system and the results are reported here.

Methods and Materials

Biological systems. The transplantable mammary adenocarcinoma 16/C (mam ad 16/C) was derived from a spontaneous mammary tumor that arose in a C3H mouse in 1974. The tumor line has been routinely maintained by s.c. implantation of tumor fragments in C3H female mice. Fragments from at least three donor tumors from a single

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passage group of C3H mice were pooled for implantation of the tumors in B6C3F1 (C57BL/6xC3H) mice. Tumor growth was followed by caliper measurements of two perpendicular diameters and tumor weight was estimated from the formula for a prolate ellipsoid, i.e., 1/2 (length $\times$ width^2).

Studies were initiated either on a designated day following tumor implantation or on a day when there were sufficient tumors within a given range of calculated weights to conduct the study. Mice were randomized to control and treated groups either immediately after tumor implantation or after staging of tumors to meet the specifications of the study.

Additional details of the tumor history and biological techniques have previously been reported by Corbett et al. [2] and Simpson-Herren et al. [18].

Drug treatment. L-PAM (NSC 8806) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, NCI, NIH, DHHS, and from Sigma Chemical Co., (St. Louis, Mo). The drug was given in H_2O on a body weight basis at the doses and on the schedule indicated for individual studies. Treatment solutions were prepared immediately prior to use and were given i.p. unless otherwise indicated.

Lissamine green (LG), a triphenylmethane dye used to delineate tumor regions of high and low perfusion [5, 11], was administered at doses of 25–50 mg/kg and was usually admixed with L-PAM at appropriate concentrations. LG is a relatively nontoxic dye that colors the interstitial fluid but does not enter living cells [6]. For some studies, LG was given prior to or following L-PAM, as indicated.

^{14}C-Antipyrine (50 mCi/mmol) was obtained from Amersham (Arlington Heights, Ill) and administered at a dose of 0.63 μCi/g body weight with 50 mg/kg LG. Mice were sacrificed 1 min after dosing.

Sample collection and preparation. Mice were anesthesized with diethyl ether 30 min after administration of L-PAM and LG. A single, terminal blood sample was collected from the axillary region into a tube containing heparin. Plasma was obtained after centrifugation of cooled samples and immediately extracted with 4 volumes of methanol. Portions of each extract were assayed for L-PAM and LG.

Tumors were excised, immediately frozen on dry ice, then dissected on the basis of relative coloration within each tumor, and the concentration of dye (and L-PAM) was later quantitated. As a result, the concentration of dye, particularly in regions of "low" perfusion, varied from tumor to tumor. Tumor tissue that had a grossly unhealthy or necrotic appearance was discarded to provide a more realistic assessment of the drug concentrations in regions that included viable cells. Each region was homogenized in 4–9 volumes of methanol and then processed as described for plasma.

The recoveries of L-PAM from plasma and the regions of high (HP) and low perfusion (LP) in tumors were 90%, 107% and 102%, respectively.

Sample analysis. HPLC analyses were accomplished with a Waters Associates (Milford, Mass) high pressure liquid chromatograph equipped with a Model 6000A high pressure delivery pump, a Model 441 UV absorbance detector, a Model 710 automatic sample injector (WISP), and a Model 730 data module. The conditions for the analysis of L-PAM and calculation of elimination half-life ($t_{1/2}$) were as previously described [18]. Although there was a minor absorbance peak for LG in the UV region, it was well resolved from the L-PAM peak and did not interfere with the analysis. LG was measured spectrophotometrically at 630 nm using a Beckman DU-2 spectrophotometer.

Bioassay of tumors. To determine whether viable cells were present in the LP regions, 50 mg/kg LG was given to mice bearing 13-day tumors ranging from 1–2 g. Tumors were harvested and dissected into LP and HP regions 30 min later. Tumor tissue from ten mice was pooled, finely minced in 4 parts of physiologic saline, placed in a hand-held Potter-Elvehjem tissue grinder, and subjected to three or four strokes of the plunger. Of the 20% weight/volume brei, 0.5 ml was implanted in the axillary region of recipient mice. Tumor growth was monitored by caliper measurements.

Results

Perfusions of tumors

Following an i.p. injection, LG was rapidly absorbed and turned the whole animal green except where there were barriers to perfusion. When mice were sacrificed at 30 min after injection of the dye, the tumors ranged in appearance from solid green, solid pink, and marbled (streaks of green and pink) to regions or nodules of green or pink. These mam ad 16/C tumors infrequently exhibited the pattern described by Goldacre and Sylvén [6], where the stained region was effectively a shell surrounding an unstained core. Very small tumors (<0.1 g) were likely to be solid green, and tumors in the range of 0.1–0.5 g were likely to be marbled with larger tumors showing distinct regions or nodules of stain. However, the described variations in staining were observed at each stage of growth. Examples of variation in staining may be seen in Figs. 1 A and 1 B.

To confirm that LG distribution was an indication of blood flow, tumor-bearing mice were given simultaneous i.v. doses of LH and ^{14}C-antipyrine, a classic blood flow indicator [1, 16]. The mice were sacrificed 1 min after dosing and tumors were removed for dissection into lightly and darkly stained regions. The samples were analyzed for total radioactivity and LG content and the results are shown in Fig. 2 A. An excellent correlation (r = 0.99) was found between the distribution of ^{14}C-antipyrine and LG stain in the tumors, indicating that LG could be used to delineate regions of high (HP) and low (LP) blood flow or perfusion.

In a second study, LG and L-PAM were given i.p. and, after 30 min, tumors were removed for dissection into lightly and darkly stained regions. These samples were then analyzed for L-PAM and LG content. The results are presented in Fig. 2 B. There was a good correlation (r = 0.94) between the concentration of LG and L-PAM. It should be noted that the measured concentrations were mean values for the region and did not necessarily reflect the concentration in the microenvironment of the cells.

Cell viability in regions of low perfusion

The presence of cells in the LP regions that were capable of continuing or reinitiating tumor growth was demon-

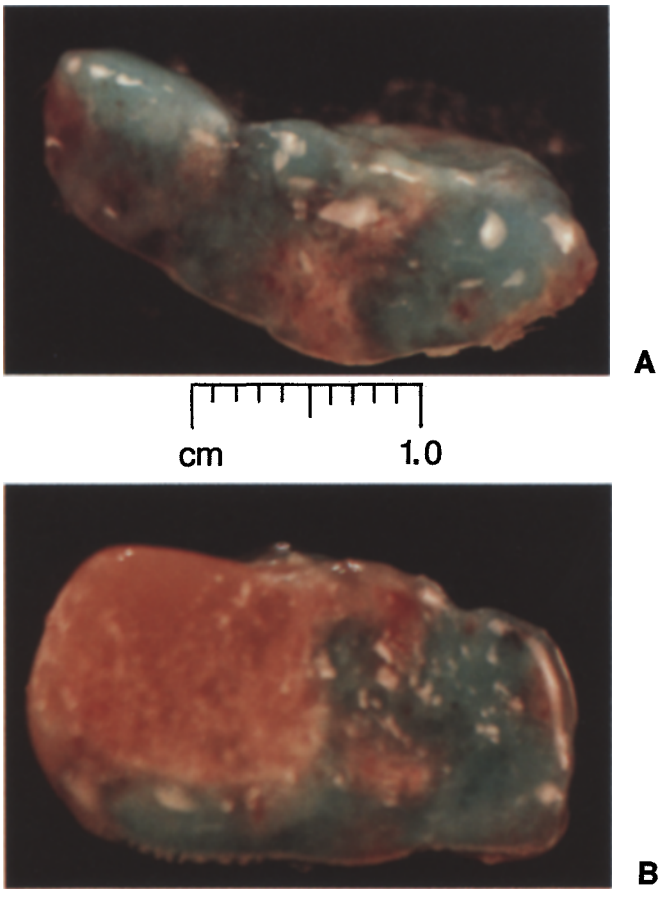

Fig. 1. Mam ad 16/C tumors 30 min after i.p. administration of LG. Regions of obvious necrosis were not dissected prior to photography

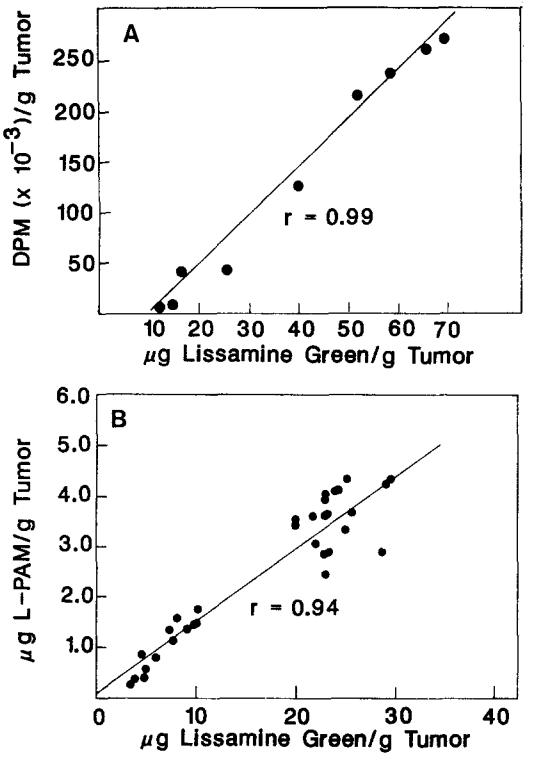

Fig. 2. **A**, Correlation between the distribution of ^{14}C-antipyrine and LG in mam ad 16/C tumors 1 min after i.p. administration. **B**, Correlation between the levels of LG and L-PAM in different regions of mam ad 16/C tumors measured 30 min after administration of LG (50 mg/kg) and L-PAM (10 mg/kg)

strated by comparative bioassay of the HP and LP regions from the same tumors. Following the implantation of brei from either HP or LP regions into the axillary region of new mice, the median time for tumors to reach 0.5 g was similar in the two groups within the limits of accuracy of the bioassay procedure (Fig. 3). The data from this study and similar studies (not shown) clearly demonstrate the presence of viable tumor-producing cells in the regions with low levels of LG and L-PAM.

Regions of low perfusion in tumors

Multiple regions or nodules of light and dark stain with gradations of color in between were seen on the periphery or on the cut surfaces of mam ad 16/C tumors. The regions of low perfusion indicated by the LG in the mam ad 16/C did not correspond to the regions of low vascularity primarily in the center of transplanted C3H mammary tumors previously described by Saeki et al. [15]. To estimate the percentage of each tumor that would be exposed to low levels of drug, the lightly and darkly stained regions were dissected and obviously necrotic tissue was discarded. The tissue from LP and HP regions was then weighed and the percentage of LP region was calculated. The data for individual tumors, both single and bilateral implants, are shown in Fig. 4. In 22 of 120 (18%) tumors analyzed, only minimal regions of low perfusion (<10% of the tumor weight) were observed. These were usually small tumors

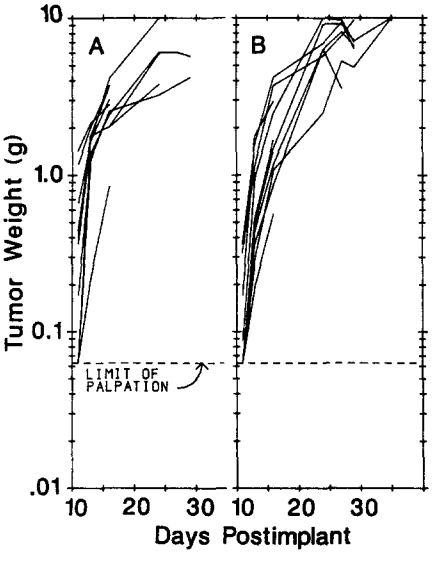

Fig. 3. Growth curves for tumors following s.c. implantation of 0.5 ml 20% weight/volume brei prepared from LP (**A**) or HP (**B**) regions of donor mam ad 16/C tumors. Ten tumors were monitored for each group, but several curves were superimposed in (**A**)

that were solid green or marbleized and the latter regions could not be readily separated. In the remaining 98 tumors, the LP regions ranged from >10% to essentially 100% of the tumor weight with large variations at all tumors ages.

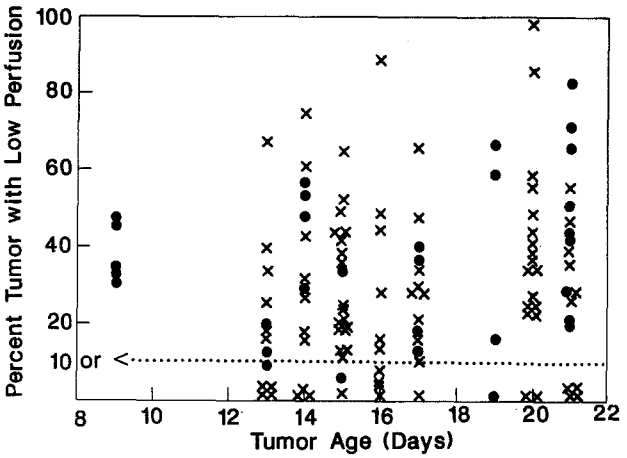

Fig. 4. Percentage of individual mam ad 16/C tumors, with low levels of perfusion delineated by LG. Values of 10% or less were not quantitated. ●, single tumors per host; X, individual tumors from mice with bilateral implants

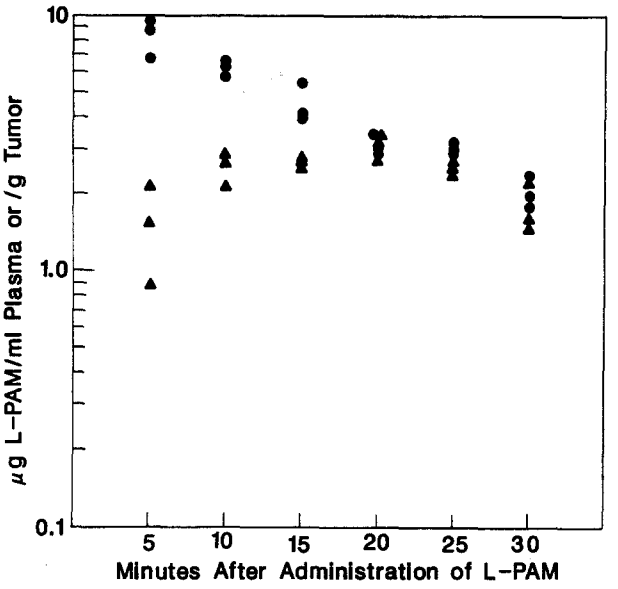

Fig. 6. Uptake of L-PAM (10 mg/kg i.p.) into plasma and tumors of mice bearing 18-day, 1- to 2-g mam ad 16/C tumors. ▲, tumors; ●, plasma

Effects of L-PAM treatment on perfusion

To determine the effect of treatment on perfusion, regions of HP and LP were estimated in single and bilateral implants 4 days after the first, second, or third dose of L-PAM. In 44/78 tumors, either the LG regions were minimal (i.e., < 10% of the tumor weight was lightly stained) or the tumors had regressed to a size (< 100 mg) where dissection into HP and LP regions was not feasible (Fig. 5). In the remaining 34/78 tumors, perfusion appeared to be as variable as that observed in the nontreated tumors.

Uptake and elimination of L-PAM from tumors

The rate of uptake of L-PAM into plasma and tumors was compared in samples taken at intervals from 5–30 min after i.p. administration of the drug. The results are shown in Fig. 6. The peak concentration of L-PAM occurred prior to 5 min in plasma and at approximately 20 min in the tumor. At 30 min, drug concentrations in tumor and plasma were similar and the fluctuations that might be introduced by small differences in sampling time were reduced.

The rate of elimination of L-PAM was compared in tumor and plasma as a function of tumor age and treatment status. The results are shown in Table 1. In animals bearing 9-day tumors (< 0.35 g), the elimination $t_{1/2}$ was 21.5 min for the tumor and 23.3 min for the plasma. In animals bearing older and larger tumors, the $t_{1/2}$ calculated for the HP areas of the tumors (43.7–49.6 min) was longer than the plasma $t_{1/2}$ (28.4–29.6 min). There was more variation in the $t_{1/2}$ calculated for tumors in previously treated mice than in previously nontreated mice. Whether or not the $t_{1/2}$ could be correlated with residual tumor burden, response, or time from last treatment could not be determined from these data.

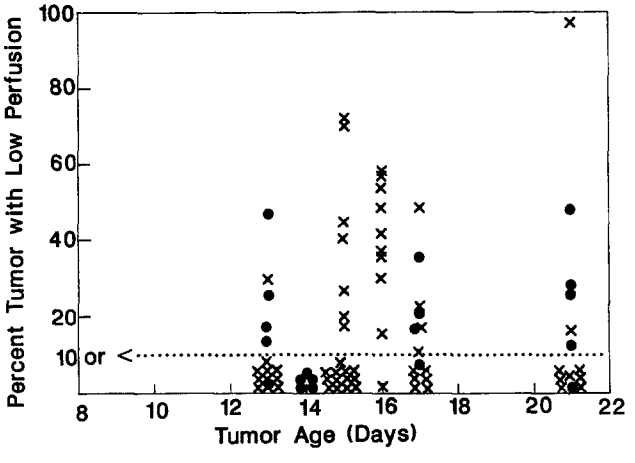

Fig. 5. Percentage of individual mam ad 16/C tumors with low levels of perfusion delineated by LG 4 days after the first, second, or third dose of L-PAM (10 mg/kg). Values of 10% or less were not quantitated. ●, single implants; X, individual tumors from mice bearing bilateral implants

Table 1. Half-life of elimination of L-PAM from plasma and tumors in previously nontreated mice and in mice previously treated with L-PAM

Status	Tumor age (days)	Tumor size (g)	Dose L-PAM (mg/kg)	Elimination $t_{1/2}$	
				Plasma	Tumor
Nontreated	9	0.35	12	23.3	21.5
	13	0.7–1.2	10	28.4	43.7
	19	2.0	10	26.0	46.6
	20	2.0	10	26.0	46.6
	21	2.0	20	29.6	49.6
Previously treated					
Regressing Rx days 9, 17	23	0.2	10	28.0	29.9
Growing Rx days 9, 17	23	2.0	10	30.3	36.0
Growing Rx days 9, 17, 23	28	2.0	10	24.5	41.7

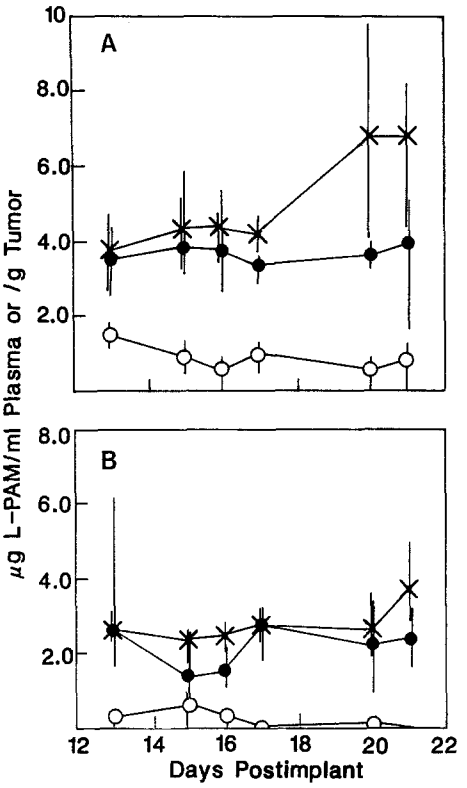

Fig. 7. Concentration of L-PAM (30 min after i.p. administration of 10 mg/kg) in plasma (X), HP (●), and LP (○) regions of mam ad 16/C tumors as a function of tumor age. **A**, no previous treatment; **b** 4 days after previous dose of L-PAM (10 mg/kg)

Distribution of L-PAM in plasma and tumors

The concentration of L-PAM in plasma and in the HP and LP regions of mam ad 16/C tumors as a function of tumor age is shown in Fig. 7A. Both the concentration and the range of values (indicated by bars) for individual tumors and mice were relatively constant from day 13 through day 17. By day 20 the plasma concentrations and variability had increased more than 75%, but the levels of drug in the tumor did not increase in proportion. The average difference between levels of L-PAM in the HP and LP regions was about threefold on day 13 and increased to tenfold or more as levels of L-PAM in the LP regions decreased with tumor age and size. The L-PAM levels in the LP regions were below the sensitivity of the assay in a number of tumors. Concurrent groups of tumor-bearing mice, studied 4 days after a previous treatment with L-PAM (10 mg/kg) (Fig. 7B), had levels of drug in the plasma and in both regions of the tumors one-third to one-half that found in nontreated mice of the same implant group. The concentration of L-PAM in the LP regions of most tumors was not detectable. On day 21, there was a slight increase of questionable significance in plasma levels, but not in the HP and LP regions of the tumors. There was an increase in the percentage of well-perfused tumors following treatment (comparison of data in Fig. 4 and 5), but levels of L-PAM in the HP and particularly the LP regions were decreased.

Discussion

The results of these studies indicate that the concentrations of L-PAM may vary as much as tenfold or more among the LP and HP regions of individual tumors. The tumor-producing cells in the LP regions, demonstrated by bioassay, probably exist in a state of oxygen and nutritional deprivation, which might be expected to reduce their sensitivity to chemotherapeutic agents directed toward proliferating cells. The low levels of drugs found in the LP regions, the presumed reduction in sensitivity of these cells to chemotherapy, and the variable percentages of individual tumors that were poorly perfused are almost certainly major factors in the variable response of individual tumors to drug treatment. In fact, the variation due to uneven perfusion of tumor regions may be an adequate rationalization for variable tumor response, although other factors may be contributory.

The levels of L-PAM achieved in the HP regions were in the same range as the concentrations reported to reduce viability of L1210 tumor cells by 50% (1–3 μg/ml for 0.5–1.0 h [13, 24]) or to inhibit the refractory pancreatic 02 cells to 50% of control (3.6 μg/ml for 24 h [23]). The actual duration of exposure of the cells to L-PAM, the active form of the drug, was dependent on the rate of hydrolysis under the conditions of culture, and direct comparison with in vivo data is questionable.

The presence of stained and unstained streaks as much as 1 mm in width within the HP regions of the mam ad 16/C tumors suggests that the variation in perfusion of drug to the microenvironment of the cells even within the HP regions may also be significant. Folkman and Hochberg [4] have suggested that 1–2 mm may be the practical limit of perfusion through tissue. Sutherland et al. [20] have reported that adriamycin failed to penetrate to the center of multicell spheroids 0.5–1.0 mm in diameter. The penetration characteristics of L-PAM have not been described, but limited data suggest that adriamycin and L-PAM are distributed similarly in the mam ad 16/C tumors (Simpson-Herren, unpublished results).

Drug levels in plasma are often used to monitor the course of chemotherapy, but there is little information on the relationship between drug levels in plasma and tissues. Of importance to efforts to monitor treatment is the observation that measurement of plasma L-PAM concentrations at 30 min after i.p. administration provided a reasonable estimate of the simultaneous concentration of drug in the HP regions of the tumors until the latter were very advanced. By day 20, levels of L-PAM in the HP regions were no longer proportional to plasma volume of the tumor [8] or to dilution factors introduced by increased distances between capillaries [21]. More likely, the L-PAM concentration was diluted by pools of blood in the tumor isolated from the general circulation [21, 22]. Furthermore, the concentrations of L-PAM in the plasma and in the HP regions of tumors were lower by 30%–50% in previously treated mice than in mice bearing tumors of similar age but with no previous treatment (Fig. 7). The highest tumor levels were observed following the initial dose, with a reduced level following the second dose. No further reduction occurred with the third and fourth dose in the protocol.

In a related study on the use of systemic dyes to demonstrate perfusion, Lewis et al. [9] have reported higher concentrations of ^{169}Yb microspheres and ^{86}RbCl in regions of two hamster tumors stained by a vital dye than in unstained regions. These authors also reported that 82% of the mass of amelanotic melanomas was unstained in con-

trast to 13% of the mass of reticulum cell lymphosarcomas. Tumor-to-tumor variations within each tumor line were apparent in the data but were not discussed in the report.

The distribution of isotopically labeled sulfur mustard and thymidine has been studied in relation to staining of Walker 256 tumors by LG [14]. The highest concentrations of the isotopes were found in stained, peripheral regions of the tumors, but labeled material, not necessarily the labeled sulfur mustard or thymidine, was found in the unstained central core. Penetration into the unstained region was reported to increase with time. That we saw no evidence of increasing levels of L-PAM with time in the unstained regions of the mam ad 16/C tumors may be attributed either to the fact that the HPLC assay was specific for L-PAM (the active form of the drug rather than metabolites or hydrolysis products) or to the diffusion characteristics of the L-PAM.

These studies clearly demonstrate the magnitude of the drug delivery problem and the tumor-to-tumor variability in perfusion in the mam ad 16/C tumors. A report on efforts to improve the drug distribution is now in preparation.

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