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Cell Survival as a Determinant of Tumor Cure for Rat 9L Subcutaneous Tumors Following Microwave-induced Hyperthermia*

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Abstract—*The relationship of cell survival to tumor cure after local hyperthermia treatment was studied in subcutaneous 9L rat tumors. Tumors weighing* 0.2–0.4 g *were heated to* 42.5, 43.0, 44.0 *and* 45.0°C *by local exposure to* 2450 *MHz microwaves. Cell survival data was obtained by an* in vivo *to* in vitro *colony forming technique and a cell survival curve was constructed for each temperature as a function of exposure duration* (0–180 min). *Cell survival followed a simple exponential function with an increasingly steeper slope as temperature increased. At* 44°C, *it was observed that cells from large tumors* (1.0–1.4 g) *were inactivated at the same rate as those from small tumors. When tumor response was monitored in the small tumors for* 90 days *following treatment, a direct correlation between the percentage of tumor cures and time at* 44°C (0–60 min) *was observed; therefore, at* 44°C, *tumor cure was exponentially related to cell survival in this range. However, when approximately the same cell survival was obtained with* 3 *other temperature–time regimens, the resulting percentage of tumor cures was not the same. These results indicate that while cell survival is related to tumor cure, it is probably not the primary determinant of tumor response following local hyperthermia in these 9L subcutaneous tumors.*

INTRODUCTION

HYPERTHERMIA is presently being used and tested clinically as an adjunct to other therapeutic modalities for treating tumors. Considerable experimental work has been done on cells in culture to determine both the mechanism of action of hyperthermia and those parameters which influence the effectiveness of heat in reducing cell survival, such as pH, glucose concentration, membrane composition and position in the cell cycle[1, 2]. To usefully transfer this experimental data to the clinic, the relationship of *in situ* cell survival to tumor response following heat treatment must be elucidated.

To correlate cell survival and tumor response, both must be evaluated in the same tumor model. There is a limited amount of data correlating these two responses after treatment with X-rays or cytotoxic drugs [3]. The 9L intracerebral rat tumor has been used to show that cell survival is a determinant of increases in life-span following treatment with X-rays [4] or BCNU [5]. In only two tumor models have both tumor response and cell survival been investigated following heat treatment [6–8]. However, it is also important that the relationship between cell survival and tumor response be demonstrated not only at one temperature, but that it also be verified at other temperatures to ascertain that cell sur-

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vival is the controlling factor in tumor response. Therefore, we undertook a systematic study of the relationship between cell survival and tumor cure after treatment of 9L subcutaneous tumors in rats with local microwave-induced hyperthermia to ascertain if cell survival is the determinant of tumor cure.

MATERIALS AND METHODS

Tumor model

9L cells derived from an *N*-methyl-nitrosourea-induced rat brain tumor [9] were grown in tissue culture using Eagle's Basal Medium (BME) containing 10% fetal calf serum (FCS), as previously described [10]. 9L/Ro cells were trypsinized from 2-day exponential cultures and adjusted to a concentration of 1×10^7 cells/ml of Hank's balanced salt solution (HBSS). A volume of 0.1 ml of the cell suspension was injected subcutaneously into the inguinal region of adult male Fisher 344 rats (Charles River Breeding Laboratories, Wilmington, MA, U.S.A.). The TD_{50} for 9L tumors in this location was $\simeq 3 \times 10^4$ cells. The tumor weight doubling time was 48.2 ± 1.5 hr for the first 13 days after transplant and 109.4 ± 3.6 hr for days 14–26 [4]. Tumors were heated at a weight of 0.2–0.4 g (10–12 days after implant) or 1.0–1.4 g (14–16 days after implant) and then either excised immediately for the *in vivo* to *in vitro* colony-forming assay, or observed daily for 30 days and twice weekly for the next 60 days. Animals were killed when the tumor reached a weight of >5 g and tumor cures were recorded when no palpable mass was observed for at least 30 days. The treatment groups evaluated for tumor cures contained 6–10 animals per group.

In vivo *to* in vitro *colony-forming assay*

The *in vivo* to *in vitro* colony-forming assay for the 9L rat brain tumor has been previously described in detail [11, 12]. The procedure for the subcutaneous tumor is essentially the same. In brief, rats were killed by cervical dislocation immediately after local exposure to microwaves; the tumors were excised, placed into ice-cold HBSS and held at 4°C until all samples were collected (0–2.5 hr) (Fig. 1). Experiments were performed to show that the colony-forming efficiency of both heated and unheated tumors was not affected by holding on ice for at least 4 hr. The tumors were then weighed, minced into fragments and placed in trypsinizing flasks with 25 ml of 0.5% trypsin (Grand Island Biological Co., Grand Island, NY, U.S.A.). The flasks were then placed in a 37°C water bath and agitated with a

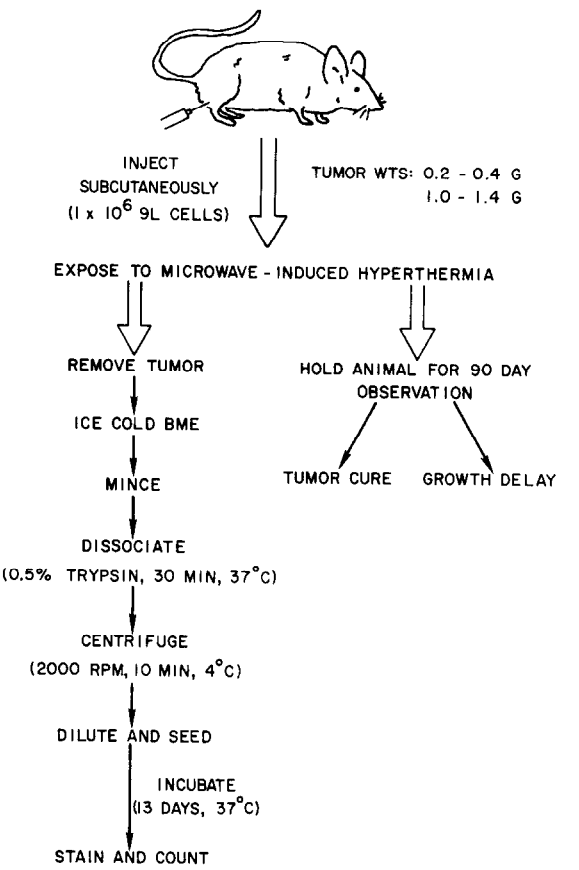

Fig. 1. Schematic illustration of the esperimental procedure used in these studies.

magnetic stir bar for 30 min. In the 30-min time interval the number of cells released per mg of tissue reached a plateau value in both unheated and heated samples. A comparison between enzymatic disaggregation procedures for both heated and unheated tumors was performed using either trypsin or a modified pronase–collagenase–DNase cocktail [13]. No difference was seen in the kinetics of cell release or cell yield/mg between cocktails. The colony-forming efficiency appeared slightly higher with the trypsin procedure.

After disaggregation the mixture was filtered through 80 μm stainless steel mesh to remove tumor debris and the filtrate was spun at 800 ***g*** for 10 min at 4°C. The supernatant was removed and the cell pellet resuspended in complete medium with 20% FCS. The suspension was counted under phase-contrast microscopy to obtain the tumor cell concentration. The effects of the disaggregation procedure were monitored using trypan blue dye exclusion; $>90\%$ of the unheated cells excluded trypan blue. The percentage of trypan blue-excluding cells decreased rapidly as the severity of heat treatment increased. Therefore, the clonogenic survival reported

was based on total tumor cells counted, not trypan blue-excluding cells.

Cells at the appropriate concentrations were seeded into either Falcon 60 mm or 100 mm petri dishes containing 5×10^4 heavily-irradiated (4000 rad) feeder cells. Feeder cells were omitted from those dishes where the experimental cell concentration was $\geq 5 \times 10^4$ cells. Three dilutions were used for each animal and 5 replicate dishes were seeded at each dilution. The petri dishes were placed in a 37°C incubator with a 5% carbon dioxide environment. After incubation for 13 days, the dishes were removed and stained with crystal violet. Only colonies of more than 50 cells were counted.

The effects of FCS concentration, feeder cell concentration and incubation duration on this assay were studied for both heated and unheated cells. These parameters were optimized so that day-to-day technical variability would not affect the number of colonies formed [14].

Heating procedure

Rats were injected with sodium pentobarbital (50 mg/kg) intraperitoneally before heating the tumor. A booster anesthetic dose of 6 mg was given when the heating duration exceeded 60 min. The heat in this experiment was induced by local microwave exposure. The microwaves were produced by a variable power (0–25 W) 2450 MHz Radarmed 12T 32 generator (ELMED, Inc., Addison, IL, U.S.A.) and transmitted through a coaxial cable to a wave-guide isolator and bi-directional coupler, where both forward and reflected power were monitored by HP8481A power sensors terminated in HP434A power meters (Hewlett–Packard). After monitoring the power levels the microwaves were transmitted through the coaxial cable into either a 15 mm or 35 mm diameter rigid coaxial contact applicator (ELMED, Inc.). The smaller applicator was used in the treatment of 0.2–0.4 g tumors (maximum diameter 12 mm) and the larger applicator was used to treat the 1.0–1.4 g tumors (maximum diameter 20 mm). The heating patterns of these applicators in phantoms have been described [15].

The tumor temperature was monitored during exposure with 0.051 mm copper–constantan thermocouples at 2 locations (central and peripheral) beneath the tumor. The thermocouples were placed orthogonally to the **E** field of the applicator. Temperature was controlled by varying the power delivered to the tumor to maintain the center thermocouple reading to within ±0.2°C of the desired temperature. Exposure duration was measured from the time the tumor reached the designated temperature. The time for this temperature rise varied from 2–10 min depending on: (1) the coupling between the applicator and tumor, (2) the state of the tumor vasculature and (3) the temperature desired. Colonic temperature of the rats was monitored with a YSI 423 thermistor (Yellow Springs Instruments, Yellow Springs, OH, U.S.A.) and was rarely found to increase above 38°C.

The temperature differential between the two thermocouples was usually 0–0.5°C and the maximum difference attained was 0.7°C. Temperature gradients in the tumor under these exposure conditions were examined using a 5-thermocouple grid. When the small applicator was used to expose 0.2–0.4 g tumors, 4 out of the 5 probes gave readings within 0.7°C of the temperature at the controlling thermocouple. Only the center probe gave a temperature reading above this range [16]. The biological effects of these gradients have been systematically studied by comparing the colony-forming efficiency of small tumor volumes (4–8 mg) taken from various locations. These data show no reproducible pattern in cell survival at the various locations. Although the average cell survival at the center is lower than the remainder of the tumor, the minimum cell survival within the tumor does not always occur at the center [16].

Cell survival analysis

Surviving fraction (*SF*) was calculated in these experiments as

$$SF = \frac{\text{colony-forming efficiency} \times \text{cell yield of heated cells}}{\text{colony-forming efficiency} \times \text{cell yield of unheated cells}}$$

where colony-forming efficiency is the number of colonies formed divided by the number of cells plated, and cell yield must be taken into account because it changes with heat treatment. The data in this paper were analyzed by a linear least-square regression of the logarithmic transform of $SF = e^{-\alpha t}$.

RESULTS

The colony-forming efficiencies (%) of tumor cells from unheated rats were 16.0 ± 0.7 (standard error S.E.) for the 0.2–0.4 g tumors (small) and 17.9 ± 1.3 (S.E.) for the 1.0–1.4 g tumors (large). These values are not statistically different ($P > 0.05$). These colony-forming

efficiencies were relatively constant (range 13.9–21.4) from 3 batches of cells over a period of one year. The cell yield per mg of tumor tissue was $1.35 \times 10^5 \pm 0.08$ in unheated small tumors and $9.10 \times 10^4 \pm 0.97$ for unheated large tumors. The cell yield from heated tumors, especially at times >60 min, was decreased by as much as 75%. Therefore, all surviving fractions were corrected for cell yield to give an accurate assessment of heat damage.

Tumor cell survival was assayed immediately after exposure to local heating. Cell survival was related exponentially to time at temperature (Fig. 2, Table 1), with T_0's of 38.9, 29.3 19.0 and 9.6 min at 42.5, 43.0, 44.0 and 45.0°C respectively. It is evident that cells were increasingly more sensitive to heat as temperature increased. Although the variability of the surviving fraction between animals was at times substantial, it was of the same order as the variability reported for other *in vivo* to *in vitro* assay systems [6, 17]. Exposure duration was measured as the time at the desired temperature, thus eliminating the heating period to the designated temperature. At all temperatures other than 45°C, the y intercept of the survival curves was not statistically different from one ($P > 0.05$). The 7–10 min heating period to reach 45°C resulted in an experimentally determined survival of 40%.

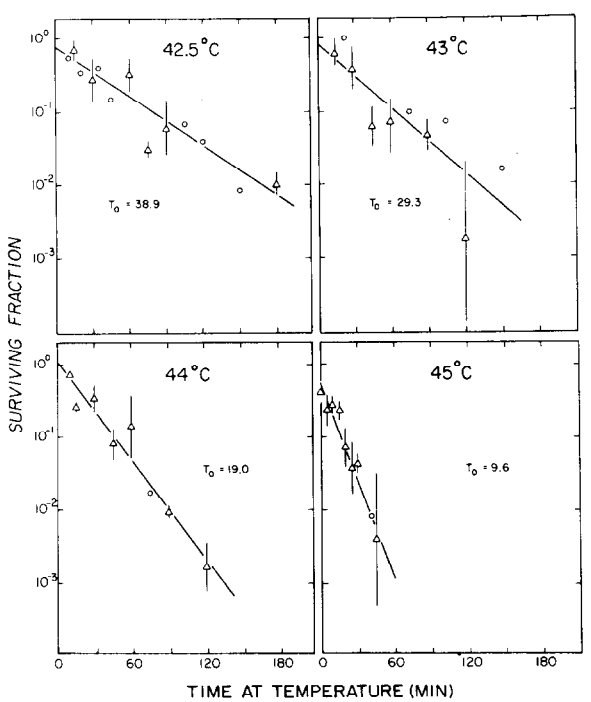

Fig. 2. Survival of 9L cells from 0.2–0.4 g tumors excised immediately after heating for various times. ○, single animals. △, the mean ± S.E. (standard error) of 2–4 animals. The lines were fitted by least-square linear regression analysis of the plotted points.

Table 1. Survival curve parameters for 9L subcutaneous tumors (0.2–0.4 g) for local hyperthermic exposure

Treatment temperature (°C)	α*(min^{-1})	T_0†(min)(± S.E.)
42.5	0.0257	38.9(35.0–43.8)
43.0	0.0341	29.3(23.9–38.0)
44.0	0.0527	19.0(17.1–21.2)
44.0‡	0.0523	19.1(17.3–21.3)
45.0	0.1040	9.6(8.8–10.6)

*Survival curves were fitted by regression analysis of the data using the simple exponential, $SF = e^{-\alpha t}$.
†$T_0 = 1/\alpha$.
‡Large tumors, 1.0–1.4 g.

In order to investigate the effects of tumor size and, perhaps, local environmental differences on cellular heat sensitivity, 2 size ranges of tumors were studied at 44°C (Fig. 3). The 2 curves had identical T_0's (19.0, 19.1), but were displaced by a surviving fraction of approximately 35%. Although the 95% confidence intervals of the two intercepts overlapped, this displacement was experimentally verified by heating the large tumors to 44°C and assaying. The resulting surviving fraction of 47% at time 0 compared favorably with the calculated intercept of 64 ± 15% (Fig. 3). This suggests that a sensitive subpopulation might exist in the large tumors.

Tumor cures were studied in 8–9 animals whose 0.2–0.4 g tumors were exposed to microwave-induced hyperthermia and observed for 90 days (Table 2). Tumor cure and

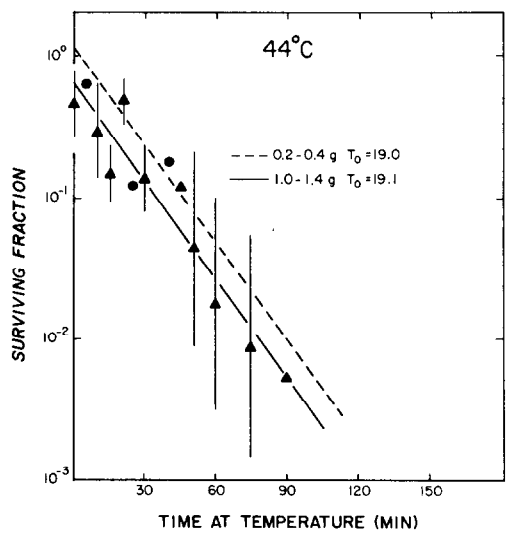

Fig. 3. Survival of 9L cells from 1.0–1.4 g tumors excised immediately after exposure to 44°C for various times. ●, single animals. ▲, the mean ± S.E. of 3–4 animals. ----, response of 0.2–0.4 g tumors to 44°C taken from Fig. 2. The line was fitted by a least-square linear regression analysis of the plotted points.

Table 2. Tumor cure of 9L subcutaneous tumors after local microwave-induced hyperthermia

Treatment Temp. (°C)	Time (min)	Surviving* fraction	No. of animals	No. tumor-free	Per cent tumor cure
44	5	0.85	8	4	50
44	15	0.50	9	3	33
44	30	0.24	8	5	63
44	45	0.11	9	7	77
44	60	0.048	9	9	100
44	90	0.010	9	8	88
44	120	0.002	8	8	100
42.5	180	0.0075	6	2	33
43	150	0.0058	7	5	71
45	30	0.0260	8	6	75
45	45	0.0050	10	4	40

*Values taken from Fig. 2.

exposure time (0–60 min) were directly related at 44°C (Fig. 4), with a TCD_{50} of $\simeq 26$ min and 100% cures by 60 min of exposure. Since cell survival was exponentially related to time at temperature, tumor cure was also related exponentially to cell survival. The cell survival level to obtain 50% tumor cures was 27%. Following 5 min at 44°C, tumor cures were disproportionately high (50%) compared to the percentage predicted by the relationship shown in Fig. 4 and Table 2.

To determine if this relationship between cell survival and tumor cure was the same for temperatures other than 44°C, 4 additional temperature–time regimens were studied. The percentage of tumor cures for 42.5°C × 180 min was 33%; 43.0°C × 150 min, 71%; 45.0°C × 30 min, 75%; and 45.0°C × 45 min, 40% (Fig. 5, Table 2). The predicted cell survival for all of these except for 45°C × 30 min was 0.5–0.8%. However, the resulting tumor cures were not

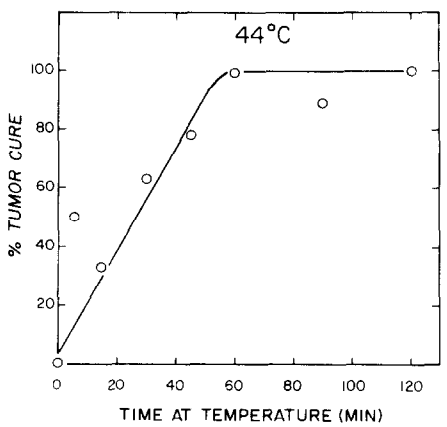

Fig. 4. Percentage of tumor cures of 0.2–0.4 g 9L tumors after various exposure durations to 44°C. Each point represents the results from 8–9 animals.

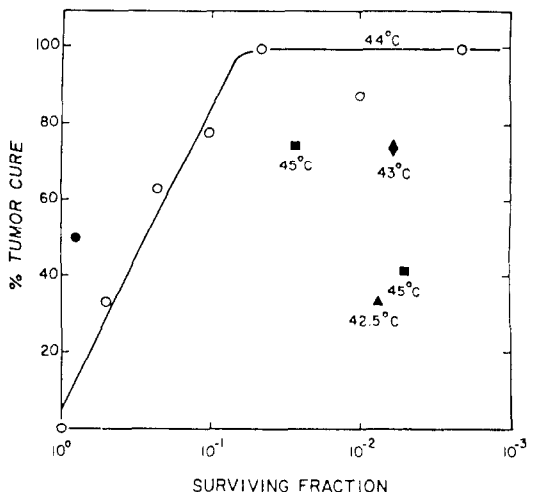

Fig. 5. Percentage of 9L tumor cures as a function of surviving fraction taken from Fig. 2. ○, ●, 44°C. ▲, 42.5°C. ◆, 43°C. ■, 45°C. Each point represents the results from 6–10 animals.

constant. At 45°C increasing the time at temperature (decreasing cell survival) resulted in a decrease in tumor cures (6 out of 8 at 30 min, 4 out of 10 at 45 min).

DISCUSSION

The intracerebral 9L rat tumor model has previously been used to study the relationship of cell survival and tumor response [4,5]. In order to use the 9L tumor to study this relationship after hyperthermic treatment, it was necessary to transplant the tumor subcutaneously. Cells immediately excised from heated tumors showed a decreased survival capacity with increasing time at a given temperature, as evidenced by the exponential decline in surviving fraction (Fig. 2). The lack of a shoulder on the survival curves may result from one of three possibilities: (1) the inability

of these cells to accumulate sublethal heat damage; (2) saturation of the ability to accumulate sublethal heat damage during the period required to heat the tumors to the designated temperature; or (3) the presence of gradients within the tumor such that a percentage of tumor cells are exposed to a higher temperature and therefore are killed with greater efficiency, masking the shoulder of the survival curve at the temperature in question. The third possibility has been studied in our lab; when the temperature at the center of the tumors was monitored, it was often found to be higher than the rest of the tumor or to overshoot the desired temperature momentarily [16]. These phenomena may be responsible for removing the shoulder of the survival curves in this set of experiments.

As can be seen by the T_0 values for the various temperatures (Table 1), the efficiency of cell kill by heat increases as temperature increases. An Arrhenius type plot of the rate of cell inactivation (α) against the reciprocal of the absolute temperature (kelvin) results in a straight line ($r^2 = 0.99$) with an activation energy of 109 kcal (Fig. 6). This activation energy is identical to that obtained for 9L cells *in vitro* at temperatures above 43°C [18]. However, the large break that occurred in the *in vitro* Arrhenius plot at 43°C is not evident in our experiments.

The efficiency of cell inactivation for the two tumor sizes exposed to 44°C treatment was identical (Fig. 3). This was somewhat unexpected in that larger tumors should have necrotic regions that would have a lower pH and oxygen concentration and, therefore, should be more sensitive to hyperthermia [19, 20]. Other investigations in our laboratory show

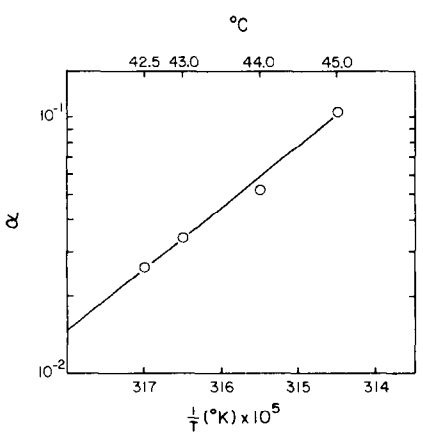

Fig. 6. Arrhenius plot of cell inactivation constants, α (Table 1), as a function of temperature. μ, Activation energy = 109 kcal.

that 0.15–1.8 g subcutaneous 9L tumors have the same X-ray sensitivity [21]. Histologically, these tumors are homogeneous, highly vascularized and have relatively few areas of necrosis. This might explain the similarities in response in the two sized tumors.

9L tumor cures were directly related to exposure duration at 44°C from 0 to 60 min (Fig. 4). This has been previously observed in a number of tumor systems (e.g., [6, 8, 22, 23]). For the 9L tumors, time at temperature was exponentially related to cell survival; this means that the percentage of tumors cured was also exponentially related to cell survival at 44°C (Fig. 5). This indicates that either cell survival is the determining factor of tumor cure or it is a covariate in the process that determines tumor cure. To distinguish between these hypotheses several different temperature–time combinations were studied to see if they fit the relationship found at 44°C. When tumor response was compared at relatively similar cell survival levels at four different temperatures (42.5°C × 180 min, 43.0°C × 150 min, 44.0°C × 100 min, 45.0°C × 45 min) the resulting tumor cures were not the same (Fig. 5, Table 2).

At the same survival level, increasing the temperature from 42.5 to 44.0°C increased the percentage of tumors cured. This might suggest that the excision assay immediately after heating may rescue cells that otherwise would die and that the proportion of the cells rescued would not be the same at different temperatures. Such an observation has been made following exposures at 43 and 44°C for 30 min in the mouse EMT-6 tumor system [7] and in the SCK mouse tumor system after a 30 min exposure to 43.5°C [24]. Our data would argue against this being the total explanation in that the 45°C exposure results in a lesser percentage of cures at the same survival level than either the 43 or 44°C exposures. A second explanation for this temperature-dependent deviance from the relationship of tumor cures vs cell survival at 44°C is that different temperatures cause different amounts of damage to the tumor's supporting vasculature. This would result in greater damage (decreased cell survival) as temperature increases. Such an affect on the vasculature has been observed [25]. Again, our 45°C data did not support this hypothesis in our tumor system.

A third explanation for this lack of a temperature-dependent correlation between tumor cure and cell survival is that cell survival is not the sole determinant of 9L tumor response to heat. The excision assay measures only cell kill resulting from heat exposure; however, this

need not be the only source of cell kill. For example, as cell killing by heat increases, the host's anti-tumor immunity might be more likely to eradicate the remaining tumor cells and produce a cure. Tumor cure, in this case, would apparently be directly related to cell survival. This possibility exists in this tumor system where the TD_{50} of 3×10^4 cells in un-immunized animals increases to $> 2 \times 10^7$ cells in animals whose tumors have been eliminated with heat treatment. However, if the effector cells of the immune response were blocked or killed as the heat treatment increased in severity, then the number of surviving tumor cells may not have the same relationship to tumor cures unless the cell kill from heat is sufficient by itself to cause a cure. This argument is supported by our data and some immunological data in the literature. First, when the time at 45°C was increased, a decrease in the number of tumor cures was observed. It is plausible that immunoreactive cells involved in the tumor response may be killed at this temperature, especially if heated for extended periods of time. Cytotoxic T lymphocytes have been reported to lose their ability *in vitro* to lyse tumor cells after prolonged heat treatment [26–28]. Another implication that the immune system may be involved in producing 9L tumor cures can be found in the increased level of cures after 5 min of 44°C exposure (Fig. 5). Stimulation of both specific and non-specific immune activities have been reported after local microwave (2450 MHz) hyperthermia of tumors [29]. If cell killing by both heat exposure and immunoreactive host cells are involved in the observed heat response, the increase in cures for an exposure of 5 min at 44°C might be expected.

Our data indicate that although cell survival measured by the *in vivo* to *in vitro* colony-forming assay is at least a covariate in the determination of 9L tumor response to heat treatment, it is not the sole determinant of the number of tumor cures. Moreover, our data indicate that the 9L tumor system provides a unique opportunity to study the effects (either stimulation or inhibition) of local microwave-induced hyperthermia on the interplay between 9L tumor cells and the host's anti-tumor immune response.

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