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Effect of 42°C Hyperthermia on Murine Normal and Tumor Tissues[1]

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ABSTRACT

The effect of one or two heat treatments at 42.0°C was studied in murine normal and tumor tissues. Early-generation isotransplants of a spontaneous fibrosarcoma, FSa-II tumors, were used. Single-cell suspension was transplanted into the mouse foot. Tumor growth time, the time required for half the treated tumors to reach 1000 cu mm from treatment day was the end point. For normal tissue studies, the foot reaction was scored according to our numerical score system. Hyperthermia was given in a constant temperature water bath. Tumors were treated when they reached an average diameter of 4 (35 cu mm) or 8 mm (270 cu mm). The 4-mm tumor responded poorly to a single heat treatment at 42.0°C, while the 8-mm tumor showed a substantial initial response, followed by the development of thermal resistance. The dose-response curve for the foot reaction was characterized by a large shoulder followed by a linear relationship. Thermal resistance developed in the 8-mm tumor following a continuous treatment of 150 min while in the foot tissue, 800 min were required before the development of thermal resistance.

The kinetics of thermal resistance were studied following the first dose of 150 min. Substantial resistance developed in both 4- and 8-mm tumors, as well as in the foot tissue, and reached maximum within 1 day. The decay of thermal resistance in the 8-mm tumor and in the foot was incomplete even at 5 days after the first heat dose, while the analysis was difficult for the 4-mm tumor because of continuous tumor growth. Comparison with treatments at 43.5°C and 45.5°C gave a conclusion that (*a*) a short single heat treatment of the 8-mm tumor at 42.0°C (below 43.0°C) resulted in a differential response between tumor and foot tissues, but a longer treatment did not; (*b*) the treatment temperature above 43.0°C was highly recommended; and (*c*) fractionated heat treatment at 42.0°C was not the choice of treatment for both 4- and 8-mm tumors.

INTRODUCTION

Biological effects of hyperthermia have been extensively studied in cultued mammalian cells and in animal normal and tumor tissues (1, 2). Some *in vitro* studies indicate that a treatment at the temperature below 43.0°C could result in a differential response between normal and tumor tissues. Namely, the cytocidal effect of hyperthermia increases with decreasing environmental pH, and this pH effect is greater at temperatures below 43.0°C (3). Numerous investigators have measured tissue pH, and found that the tumor tissue pH is lower than the normal tissue pH (4–6). This is due to extensive glycolysis in the tumor tissue, which leads to an accumulation of lactic acid and eventually reduces the tumor tissue pH (7). In these situations heat treatment below 43.0°C could result in a greater differential response than the treatments above 43.0°C. Recent *in vitro* data on the kinetics of thermal resistance (thermotolerance) at different pH levels further suggest less extensive development of thermotolerance in the low pH tissue than in the normal pH tissue (8, 9). Namely, fractionated hyperthermia may favor differential response between low pH and normal pH tissues. We have investigated the potential differential response between normal and tumor tissues in our animal system at 42.0°C. Furthermore, the response of 2 different sized tumors was compared, since the tumor tissue pH was reported to decrease with increasing tumor size (10). Tissue thermal response may be modified by the blood flow rate which alters oxygen and/or nutrient supply to the tissue. The blood flow rate may be influenced by tissue temperatures and, in the tumor, by tumor size (11, 12).

MATERIALS AND METHODS

Animals were 8- to 10-week-old C3Hf/Sed mice derived from our defined-flora mouse colony. They were kept in our animal facility where the defined-flora conditions have been maintained (13). Animals received acidified and vitamin K-fortified water and sterilized mouse pellets *ad libitum*. Early-generation isotransplants of a fibrosarcoma which arose spontaneously in a C3Hf/Sed mouse, FSa-II, were used. Single-cell suspensions were prepared by trypsinization. Namely, intact tumor tissues were trypsinized at room temperature and single cells were collected by centrifuging the supernatant. The detailed method is given elsewhere (14). Five μl of this single-cell suspension containing approximately 2×10^5 viable tumor cells were transplanted into the animal foot.

Hyperthermia was given in a water bath where a desired temperature ± 0.1°C was maintained by a constant temperature circulator. Each animal was kept in an individual holder. The tumor-bearing leg was taped on an arm of the holder and was immersed in the water bath. Temperatures in the 4- and 8-mm tumors were no less than 0.1°C below the water bath temperature (15). Animals were treated without anaesthesia when tumors reached an average diameter of 4 or 8 mm (35 or 270 cu mm).

Tumor response was studied by the TG time[3] assay where the TG time means the time required for one-half the treated tumors to reach 1000 cu mm (average diameter of 12.5 mm) from the first treatment day. Three diameters of each tumor, *a*, *b*, and *c* were measured 3 times a week, and the tumor volume was calculated by $\pi abc/6$. A tumor growth curve was drawn to determine the TG time for each tumor. The median TG time was calculated by the logit analysis method (15).

The foot reaction was scored to study normal tissue response to hyperthermia. Our numerical score includes skin reaction (score, ≤ 3.5) and irreversible bone necroses (score, ≥ 4.0). The foot reaction was scored every day between Days 8 and 14 and then twice a week until Day 35. The peak foot reaction was the mean of 3 largest scores. The details of the score system is given elsewhere (16).

At least 8 animals were used at each dose level in both foot reaction and tumor response studies, and all experiments were performed at least twice.

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[3] The abbreviations used are: TG time, tumor growth time, the time required for one-half the treated tumors to reach 1000 cu mm from treatment day; D_1 and D_2, the first and second dose, respectively.

RESULTS

The response of 4- and 8-mm tumors to a single or 2 heat treatments was studied. The 4-mm tumor responded very poorly to a single treatment at 42.0°C (Chart 1). No growth retardation followed hyperthermia of up to a treatment time of 150 min. Following a treatment longer than 150 min, an exponential relationship was observed between the TG time and the treatment time. The response of the 8-mm tumor to a single treatment was much greater than that of the 4-mm tumor (Chart 2). The dose-response curve was initially exponential with no shoulder. The prolongation of the TG time reached a plateau after a 150-min treatment, and further prolongation was observed following longer treatment. The second plateau was observed at the treatment time longer than 300 min.

A D_1 of 150 min was used for 2 dose experiments because thermal resistance apparently developed in the 8-mm tumor treated with this dose (Chart 2).

The 4-mm tumor which received a D_1 of 150 min was very resistant to the D_2 given 24 or 48 h later (Chart 1). No growth retardation was observed for the total treatment time of 450 min, indicating the development of substantial thermal resistance. The resistance appeared to have decreased with increasing time interval. The response of the tumor to the D_2 given 3 days after D_1 was comparable to the response of the 4-mm tumor to a single dose. The response of the tumor to the D_2 given 120 h after D_1 was greater than the response of the 4-mm tumor to a single treatment. It is notable, however, that tumors grew continuously following a D_1 of 150 min. The mean diameter of the tumor at the third and fifth days after D_1 was 6 and 8 mm, respectively.

The 8-mm tumor treated with a D_1 of 150 min also showed strong resistance to the D_2 given not only 24 to 48 h, but also 72 to 120 h after D_1, indicating that substantial thermal resistance developed within 1 day and did not decay completely at least for 5 days (Chart 2). Unlike the 4-mm tumor, the growth of the 8-mm tumor was inhibited for approximately 6 days by the D_1 of 150 min (growth delay time following a 150-min treatment was 6 days. See Chart 2).

In the next experiment we examined the development of thermal resistance following a small D_1 in the 8-mm tumor. A D_1 of 30 min at 42.0°C was followed by graded second doses 24 h thereafter. A D_2 ranging from 0 to 270 min failed to retard tumor growth, again indicating the development of thermal resistance (Chart 3, *top*). Chart 3, *bottom*, illustrates the kinetics of thermal resistance following a D_1 of 30 min. The D_2 was fixed as 180 min. It is clear that the resistance developed within 24 h and decayed slowly. It was difficult to examine the time interval between 2 treatments of more than 72 h because the tumor grew rapidly following this small dose (note in Chart 2 that a 30-min treatment at 42.0°C caused a tumor growth dalay of only 2 days).

Comparable experiments were performed in the mouse foot. A single dose-response curve showed a large shoulder followed by a linear relationship, and reached a plateau after a continuous heating of approximately 800 min (Chart 4). The dose-response curve was similar to that of the 4-mm tumor but apparently different from that of the 8-mm tumor. Substantial thermal resistance also developed in the murine foot. When a D_1 of 150 min was followed by graded D_2 with a time interval between 2 treatments of 24 to 120 h, the dose-response curve following the second dose was less steep than that following a single dose. The resistance did not decay completely at least for the first 5 days following the initial treatment (Chart 4).

DISCUSSION

The relationship between the TG time and the score of average peak foot reaction can be obtained from the dose-response

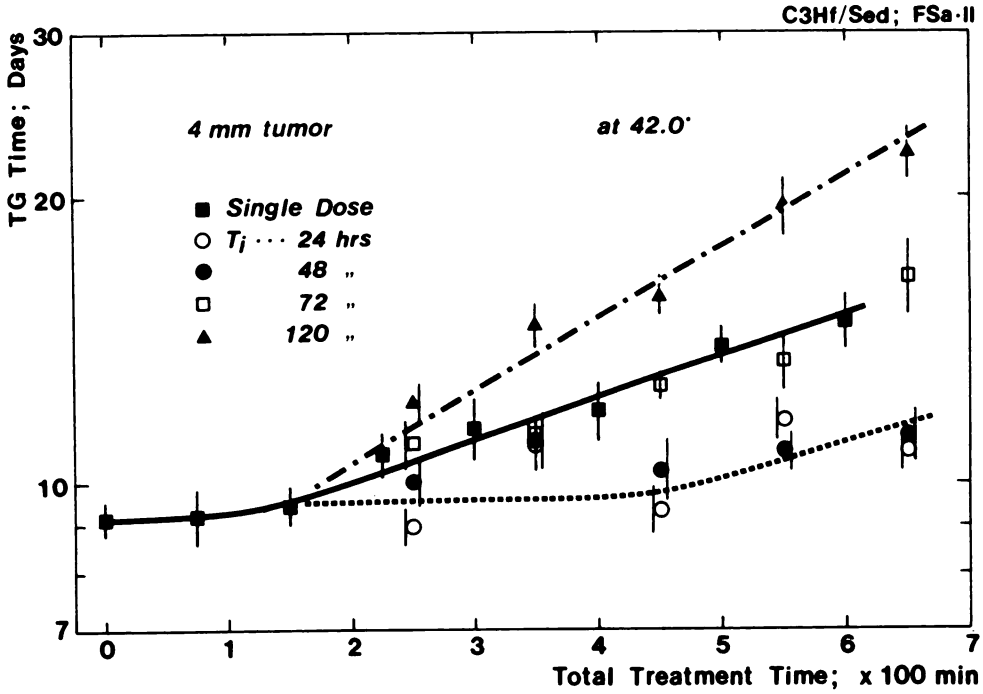

Chart 1. Dose-response curves of the 4-mm tumor following a single heat dose or 2 heat doses. The TG time is plotted as a function of total treatment time at 42.0°C. In 2-dose experiments a D_1 of 150 min was followed by graded D_2 at various time intervals between 2 treatments (T_i). *Bars*, 95% confidence limits.

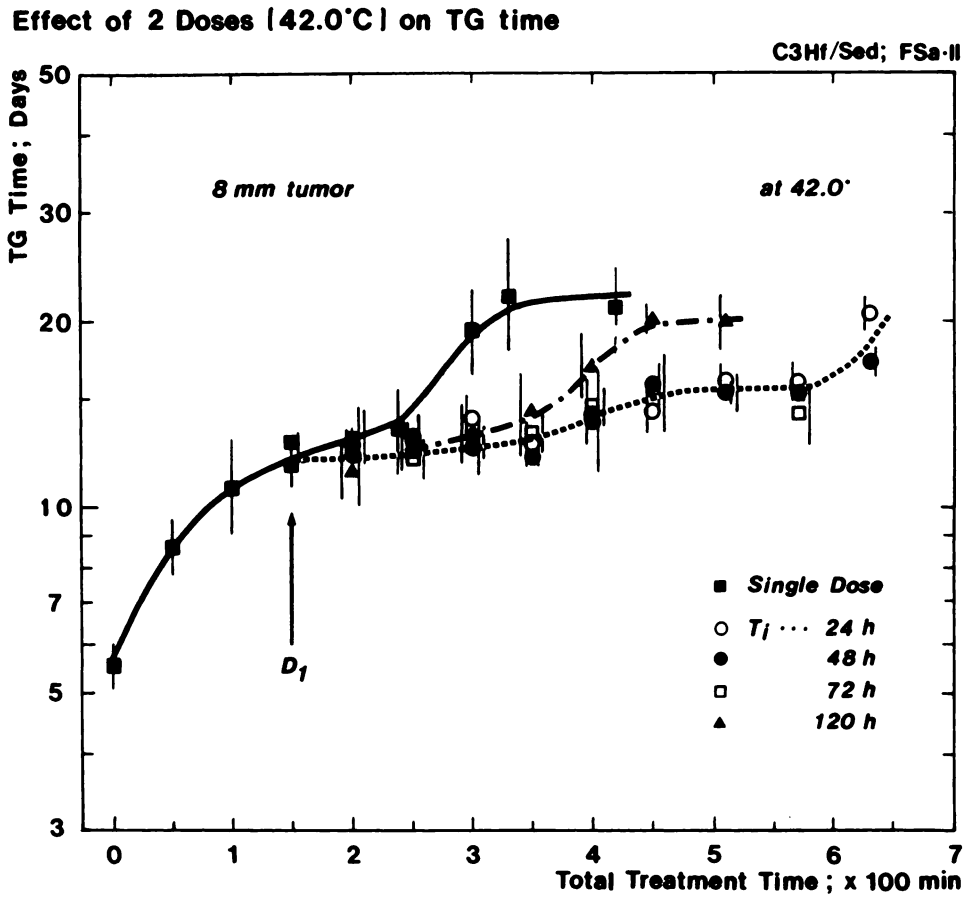

Chart 2. Dose-response curves of the 8-mm tumor following a single heat dose or 2 heat doses. The TG time is plotted as a function of total treatment time at 42.0°C. A D_1 of 150 min was followed by graded D_2 in 2 dose studies. *Bars*, 95% confidence limits. T_i, time interval between 2 treatments.

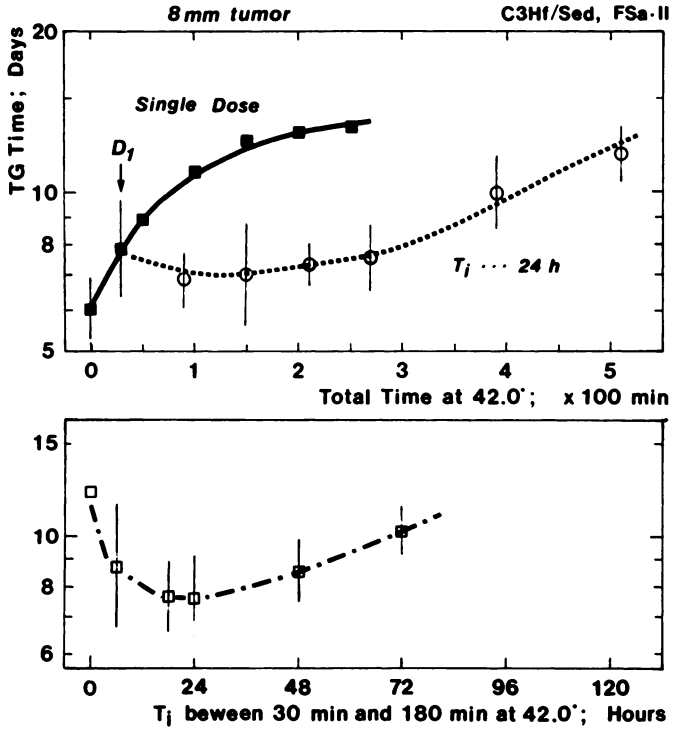

Chart 3. Kinetics of thermal resistance in the 8-mm tumor following a D_1 of 30 min. *Top*, dose-response curves following a single dose and following a D_1 of 30 min plus graded D_2 given with a time interval between 2 treatments (T_i) of 24 h. *Bottom*, TG time following a D_1 of 30 min and a D_2 of 180 min is plotted as a function of T_i between 2 doses. *Bars*, 95% confidence limits.

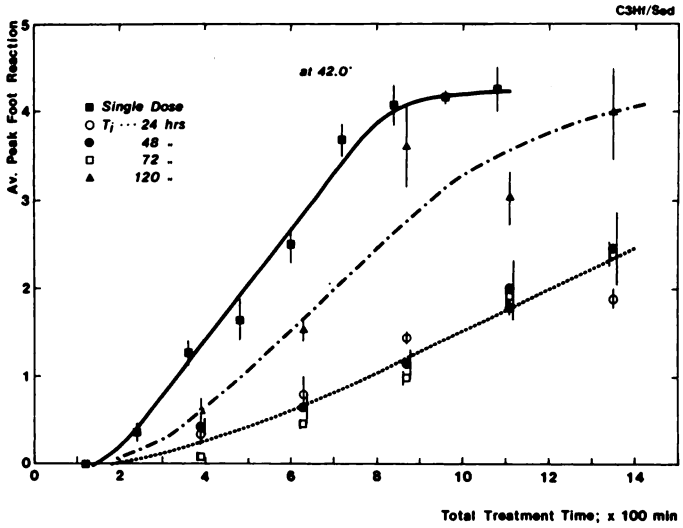

Chart 4. Dose-response curves between average peak foot reaction and total treatment time at 42.0°C. In 2 dose studies a D_1 of 150 min was followed by graded D_2 with a time interval between 2 treatments (T_i) of 1 to 5 days. *Bars*, SE.

curves for the tumor response and for the foot reaction. It may be clinically relevant to compare this type of relationship obtained at various treatment temperatures to choose a treatment temperature which results in a differential response between normal and tumor tissues.

We have studied the response of normal and tumor tissues at various temperatures. Typical dose-response curves at 43.5°C and 45.5°C are taken from previous papers (15, 17–19) and are shown in Charts 5 (tumor response) and 6 (foot reaction), together with present data at 42.0°C. The relationship between the TG time of the 4- or 8-mm tumor and the score of average peak foot reaction at each temperature was obtained from the results shown in Charts 5 and 6. These relationships are shown in Chart 7. The graph may be read as follows: for example, the treatment time which induces an averge foot reaction of 2.0 prolongs the TG time of the 4-mm tumor from 9.8 to 13 days at 42.0°C, to 18 days at 43.5°C, and to 21 days at 45.5°C. This implies that a treatment time which induces Score 2.0 reaction leads to the least tumor response at 42.0°C compared to 43.5°C and 45.5°C. In general, the TG time is shortest at 42.0°C at any level of foot reaction, indicating that 42.0°C is not a favorable choice of treatment for the small tumor. We have 3 other dose-response curves of the 4-mm FSa-II tumor treated at 45.5°C (17, 19, 20). The relationship between the TG time and the average foot reaction from these experiments fell between the relationship at 43.5°C and that at 45.5°C shown in Chart 7 (data are not shown). This indicates that the therapeutic ratios at 43.5°C and 45.5°C are comparable.

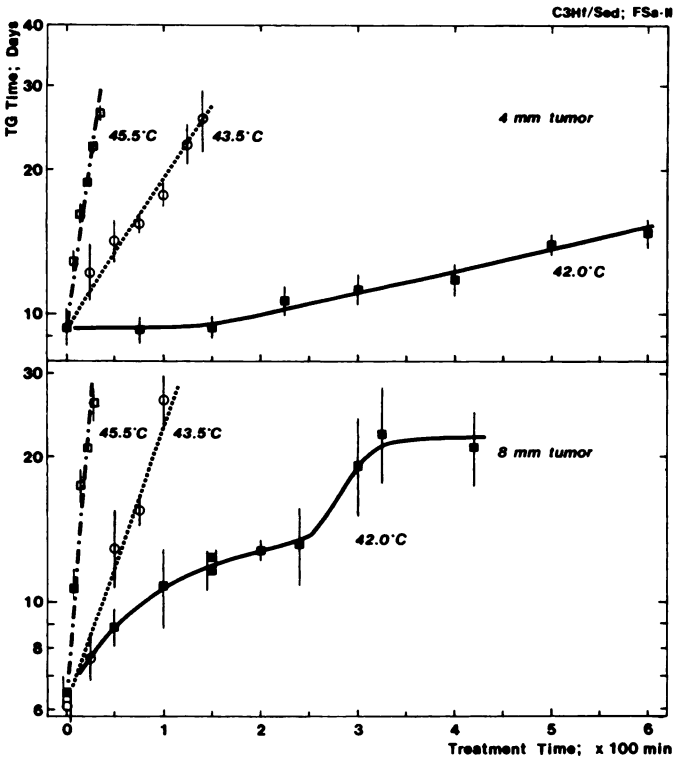

Chart 5. Dose-response curves of the 4-mm tumor (*top*) and of the 8-mm tumor (*bottom*) receiving a single heat treatment at 42.0, 43.5 and 45.5°C. The dose-response curves to 42.0°C are taken from Charts 1 and 2, while those to 43.5 and 45.5°C are taken from our previous studies (15, 19).

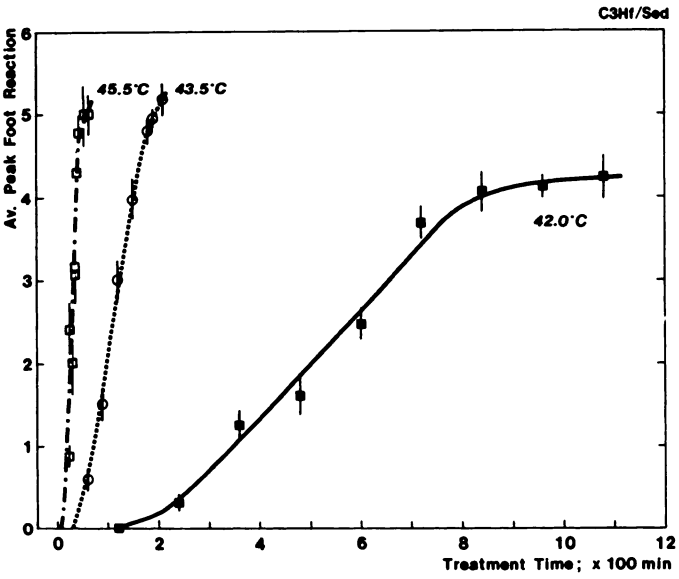

Chart 6. Dose-response curves for the foot reaction following a single treatment at 42.0°C, 43.5°C, and 45.5°C. The dose-response curve to 42.0°C is taken from Chart 4, while those to 43.5°C and 45.5°C are taken from our previous studies (15, 19).

No significant difference was observed in the relationship for the 8-mm tumor between 42.0°C and 43.5°C and between 42.0°C and 45.5°C. A slight difference was seen between 43.5 and 45.5°C below the foot reaction score of 1.0. However, in such a low-dose area large experimental variations are not unusual. In fact, we failed to demonstrate the difference in another experiment in which a dose-response curve was obtained at 45.5°C (20) (data not shown). Furthermore, in this type of analysis, it is difficult to compare the effect of a small dose which induces no foot reaction. As shown in Chart 7, *right* prolongation of the TG time from 6.2 to 11 days was induced by the treatment time which caused no foot reaction at any temperature. The failure in this comparison is due to a large shoulder found in the dose-response curve of the foot reaction. Our previous studies indicated a common activation energy for the response of the 4-mm FSa-II and for the foot reaction at the treatment temperature above 42.5°C, while the same activation energy was found for the 8-mm tumor at the temperature above 41.0°C (21, 22). The same relationship was observed for the response of the 8-mm tumor shown in Chart 5. The treatment times which induced a TG time of 10 days were 90, 37, and 9 min at temperatures of 42.0°C, 43.5°C, and 45.5°C, respectively (Chart 5). These values can be found on an Arrhenius plot with the same activation energy as previously observed for the 8-mm tumor. This indicates that a single-dose treatment of a large tumor at 42.0°C could result in the differential response between normal and tumor tissues. However, the development of thermal resistance during the treatment at 42.0°C eliminates the differential response following a treatment longer than 150 min.

The difference in the response of 4- and 8-mm tumors may be attributed to the pH effect (15). It has been demonstrated that the thermal sensitivity of mammalian cells increases with a decrease in environmental pH (3), and that the tumor tissue pH decreases with increasing tumor size (10, 23). The average pH values in the 4- to 6- and 8- to 10-mm FSa-II tumors were 7.05 and 6.92, respectively.[4] These observations may in part explain the increased sensitivity of the 8-mm tumor compared to the 4-mm tumor. Reduced blood flow in the large tumor (12) may also contribute to this observation.

It has been well demonstrated in cultured mammalian cells that the pH effect is more dramatic at temperatures below

[4] J. G. Rhee, *et al.*, unpublished data.

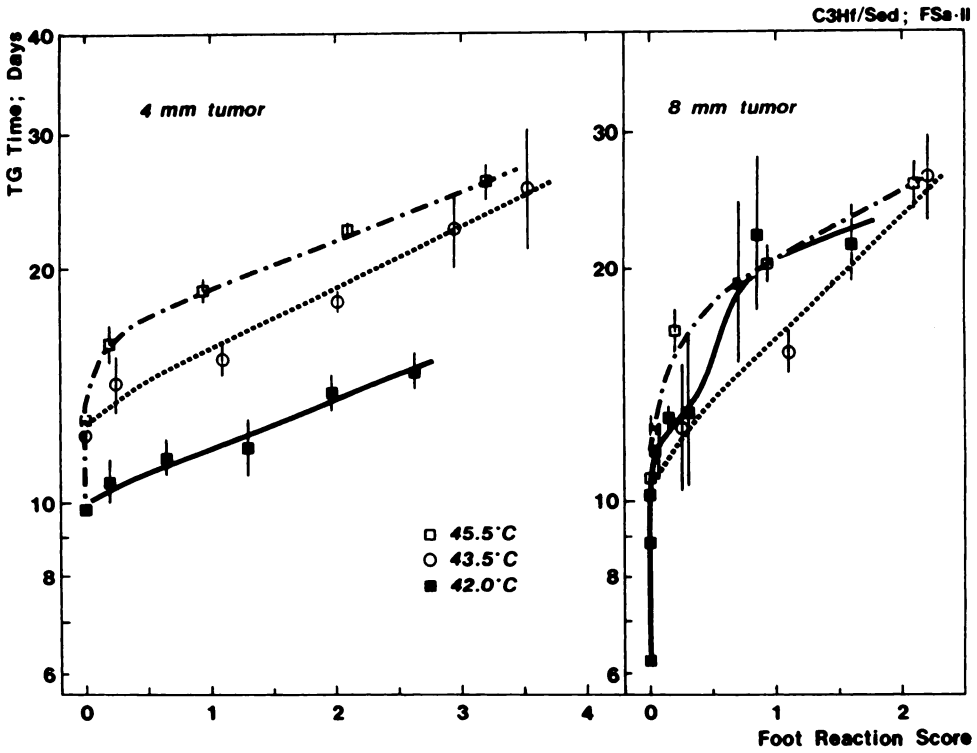

Chart 7. Relationships between the TG time and the score of average foot reaction following a single treatment given at 42.0°C, 43.5°C, and 45.5°C. *Left and right panels* show the relationship for the 4- and 8-mm tumors, respectively. Relationships at 42.0°C were obtained from the present study, while those at 43.5°C and 45.5°C were calculated from our previous studies (15, 17–19).

43.0°C compared to temperatures above 43.0°C, and increases greatly at decreasing pH (3). This feature of the pH effect suggested that the treatment of the 8-mm tumor at 42.0°C should result in a greater differential response than that above 43.0°C. Our results were only partially compatible with this suggestion (Chart 7). The differential response was only obtained following a single treatment shorter than 150 min for the 8-mm tumor, and was lost following a longer treatment. The loss is most likely attributed to nonuniform pH distribution in the tumor tissue (24). Tumors may contain a small but significant fraction of normal pH tumor cells which are resistant to hyperthermia and rapidly develop thermal resistance. The result of over 500 pH measurements indicated that 63% of 4- to 6-, and 29% of 8- to 10-mm tumor measurements were greater than pH 7.0.[4]

The second steep slope of the single dose-response curve of the 8-mm tumor (see Chart 2) may be the result of further heating which might have further reduced tumor tissue pH (11) and made tumor cells more susceptible to continuous heating. However, this did not contribute to the differential response between the 8-mm tumor and the foot tissue. Regarding the foot reaction, it is unknown why thermal resistance or plateau response did not develop during a continuous treatment for 800 min. However, it is likely that the resistance developed in the first 150 min and the observed slope is that of the resistant cells.

It has been shown in cultured mammalian cells that thermal resistance following the first heat dose develops less extensively in low pH medium as compared to normal pH medium (8, 9). This appears to contradict the development of extensive thermal resistance in the 8-mm tumor. The development of substantial resistance might be attributed to the nonuniform pH distribution in the tumor as mentioned above, or to the capability of tumor cells to develop substantial resistance in low pH conditions. Recently it has been shown that the decay of thermal resistance might correlate to the cell-doubling time (25). The volume-doubling time of the FSa-II tumor is slower at the diameter of 8 mm than at 4 mm and, in addition, the 8-mm tumor might contain more nonproliferating tumor cells than does the 4-mm tumor.

The 4-mm tumor treated with a single dose of 150 min at 42.0°C grew continuously, and the average diameter of the tumor reached 6 and 8 mm at 3 and 5 days, respectively, after the first treatment. This difference in the tumor size could explain the thermal sensitivity of the tumors to the D_2 given 72 or 120 h after the D_1.

The present study indicated that a single heat treatment at 42.0°C to the 4- or 8-mm tumors resulted in less or equal differential response, respectively, compared to the single treatment at temperatures above 43.0°C. The only exception was observed following a short treatment given to the 8-mm tumor at 42.0°C which resulted in a differential response between normal and tumor tissues. The fractionated treatments at 42.0°C would not be recommended because of the development of substantial thermal resistance. In the 8-mm tumor substantial resistance still remained 120 h after the first dose. The 4-mm tumor treated with a D_1 of 150 min grew continuously thereafter. Recent encouraging results using low temperatures show that hyperthermia combined with some chemotherapeutic agents is effective at temperatures below 43.0°C (26, 27).

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