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EFFECT OF HYPERTHERMIA ON A NEUROGENIC RAT CELL LINE (BT_4A) IN VIVO

OLAV DAHL

Neurogenic tumours are generally quite resistant against irradiation and cytotoxic drugs at doses which are tolerable for normal tissues. Hyperthermia alone or combined with other modalities may increase the therapeutic index.

Few reports on the effect of heat alone or combined with cytotoxic drugs in culture on neurogenic tumours have been published (9L cells: ROSS-RIVEROS & LEITH 1979, HATHAWAY & ALPEN 1980, HENDERSON et coll. 1980; human glial cells: GERWECK & BURLETT 1978, RÖTTINGER & MENDONCA 1980, GERWECK & RICHARDS 1981; BT_4C cells: DAHL 1980, 1981). The in vivo data for neurogenic tumours are also sparse (SUTTON 1971, KREMKAU 1979, MAGIN & JOHNSON 1979, THUNING et coll. 1980, WALLEN et coll. 1980).

Techniques for heating of intracranial tumours (focused ultrasound: HEIMBURGER et coll. 1974, SACHS 1975, FRY 1978, LELE et coll. 1978, LELE 1980; electromagnetic radiation: HO 1979, STROHBEHN et coll. 1980, SAMARAS et coll. 1980, TAYLOR 1980, 1981; thermoseeds in radiofrequency fields: BURTON et coll. 1971, MOIDEL et coll. 1976; local perfusion of heated blood: CUMMINS et coll. 1980) are developing. Therefore, it is of general interest to know in more detail the biologic effects of hyperthermia on neurogenic tumours as a basis for the improvement of such methods.

Recently, the effect of hyperthermia alone on the neurogenic rat cell line BT_4C in culture was presented (DAHL 1980). The present report describes the effect of hyperthermia on the same cell line grown as solid transplantable tumours in the feet of BD IX rats (designated BT_4A in animals).

Materials and Methods

Rats of the inbred strain BD IX (LÆRUM et coll. 1977) aged 8 to 10 weeks, average weight 166 g, were used. The animals were housed in plastic cages and given pelleted food and water ad libitum.

Tumour line. The tumour cell line originated after a single transplacental dose (75 μg/g body weight) of N-ethylnitrosourea, administered to a pregnant BD IX rat by intravenous injection on the 18th day of gestation (LÆRUM & RAJEWSKY 1975). The dissociated fetal brain cells grown in long term culture became tumourigenic after about 200 days.

The animal tumour was produced by subcutaneous inoculation of a suspension of single cells from culture. For serial transplantation, the tumours were removed under sterile conditions, separated from the surrounding connective tissue and divided in sterile medium containing antibiotics (DAHL 1980). Tumor pieces measuring about 1 to 2 mm were inoculated through a small incision on the thigh of ether anesthetized animals, and then passed through a preformed channel down to the dorsal part of the feet. No wounding was performed over the tumour and the technique therefore permitted a

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rough evaluation of direct skin damage due to hyperthermic therapy. Animal passages 4 to 11 were used in the experiments. Tumours were found in 95 to 100 per cent of inoculated animals.

Morphology. The neuroectodermal BT_4C cell line is probably of glial origin (LÆRUM et coll.) and contains the nervous system specific protein S 100.

The histopathology of subcutaneously transplanted tumours after fixation in buffered 4% formalin and staining with hematoxylin and eosin is shown in Fig. 1. The cellular tumour predominantly has a uniform neurinoma-like appearance with abundant mitotic figures.

Heating. All animals were anesthetized in ether and then fixed in a specially constructed lucite jig (Fig. 2) which allowed the rats to lie in correct position with firm fixation of the tail by taping the tail to the jig (OVERGAARD 1980). The right tumour-bearing leg was at the same time passed through a window and kept in position with tape sticking to the hairs of the thigh without impairing the blood flow of the tumour-bearing leg.

After fixation, the fully awake rats were placed in a circulating water bath (TE 623 Hetotherm, Heto, Birkerød, Denmark) with the tumour immersed at least 1.5 to 2 cm below the surface. The water bath temperature was within ±0.03°C of the preset value during the treatments (measured by a Kalorimeter-Thermometer, range 40–50°C, graduation interval 1/100, Karl Schneider & Sohn, Wertheim am Main, Germany). The temperatures quoted are those of the water bath. The time was measured from immersion to removal of the rats from the water bath. The control animals were fixed in the same manner and treated in a water bath at ambient temperature for one hour.

When the intratumoural temperature was measured by thermocouples (copper-constantan, Tarkan W+W Recorder 900, Basel, Switzerland with Frigistor Reference Chamber) the tumours examined reached within 0.2°C of the temperature in the water bath at 42.0 and 43.0°C. The intratumoural temperature was then stabile. At 44.0 and 45.0°C the intratumoural temperature first reached a top and then decreased after 4 to 5 min until stabilizing 0.5 to 0.8°C below the water bath temperature.

Growth evaluation. In preliminary experiments (unpublished data) using the glioma line BT_2A (LÆRUM & RAJEWSKY) the subcutaneously implanted tumours grew significantly (unpaired Student's t-test, $p<0.01$) slower in the foot compared with the thigh and the neck: Relative volumes after 26 days were 1.0, 5.4 and 8.1, respectively. A similar regional difference was also apparent for the BT_4A cell line.

Two weeks after implantation, tumours measuring between 5 and 12 mm (smallest and largest diam-

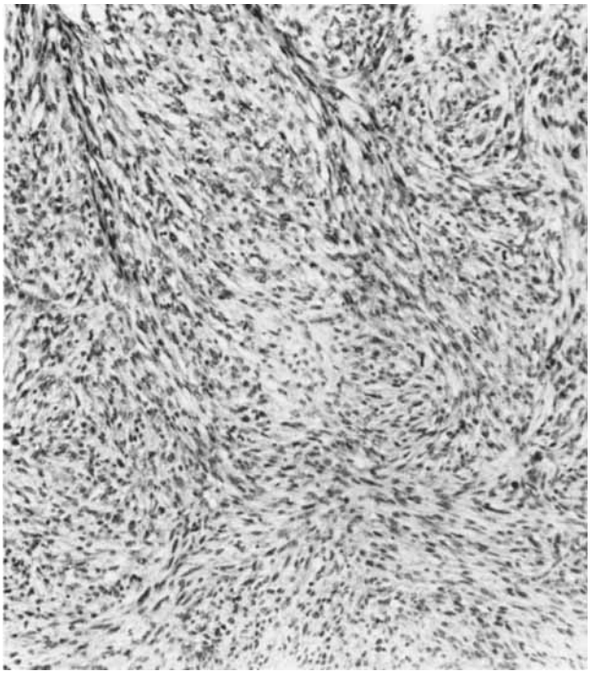

Fig. 1. Photomicrograph of a solid BT_4A tumour grown subcutaneously in the foot of a BD IX rat. Typical neurinoma-like growth. Hematoxylin-eosin stain. ×120.

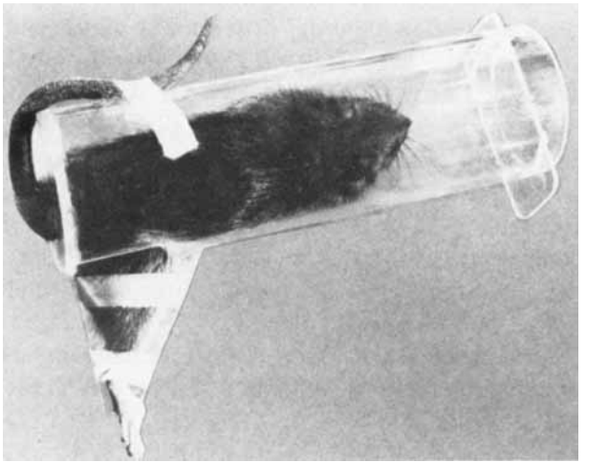

Fig. 2. BD IX rat fixed by firm taping of the tail to the lucite jig. Tumour-bearing leg in treatment position. The fixation permitted simultaneous treatment of 6 animals in a circulating water bath.

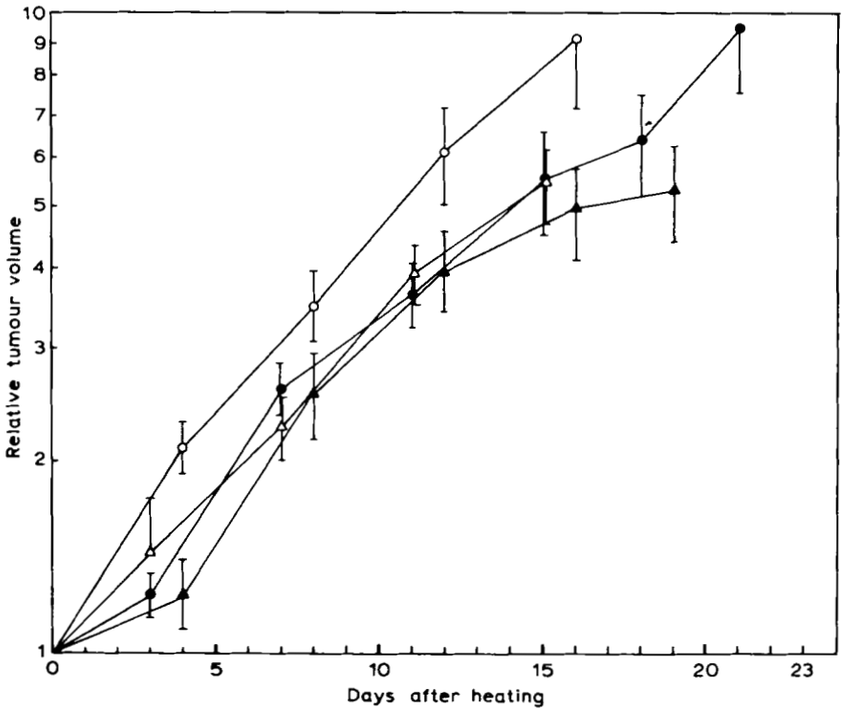

Fig. 3. Growth curves for solid BT_4A tumours treated by immersion of tumour-bearing legs in a water bath at 42.0°C for different times. Control, ambient temperature (○), 2 h (●), 6 h (△), 12 h (▲). Each curve is the mean of groups of 9 or 10 rats. Vertical bars: ± SEM.

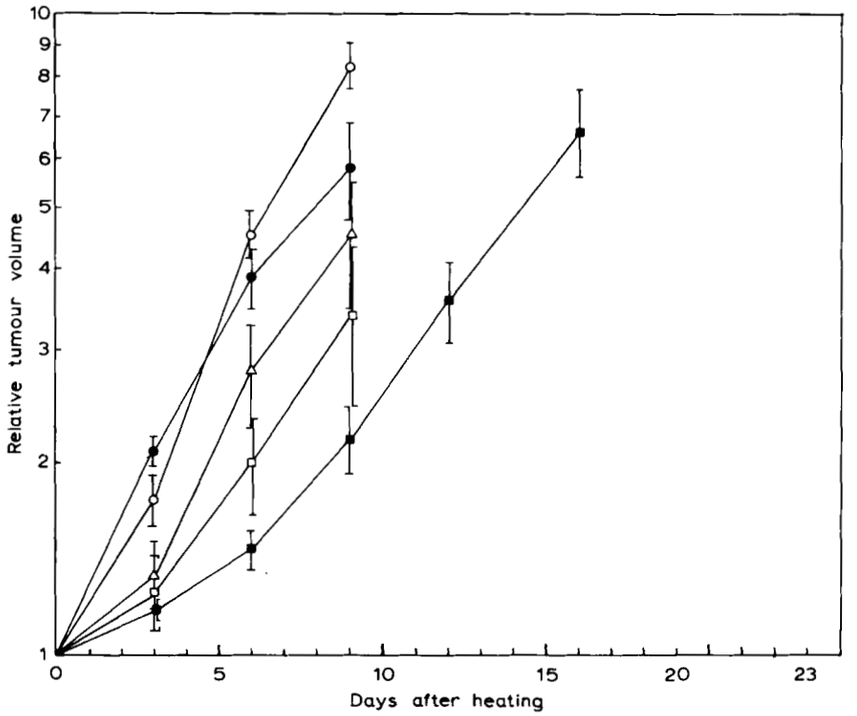

Fig. 4. Growth curves for solid BT_4A tumours treated by immersion of tumour-bearing legs in a water bath at 43.0°C for different times. Control, ambient temperature (○), 30 min (●), 60 min (△), 90 min (□), 120 min (■). Each curve is the mean of groups of 10 to 14 rats. Vertical bars: ± SEM.

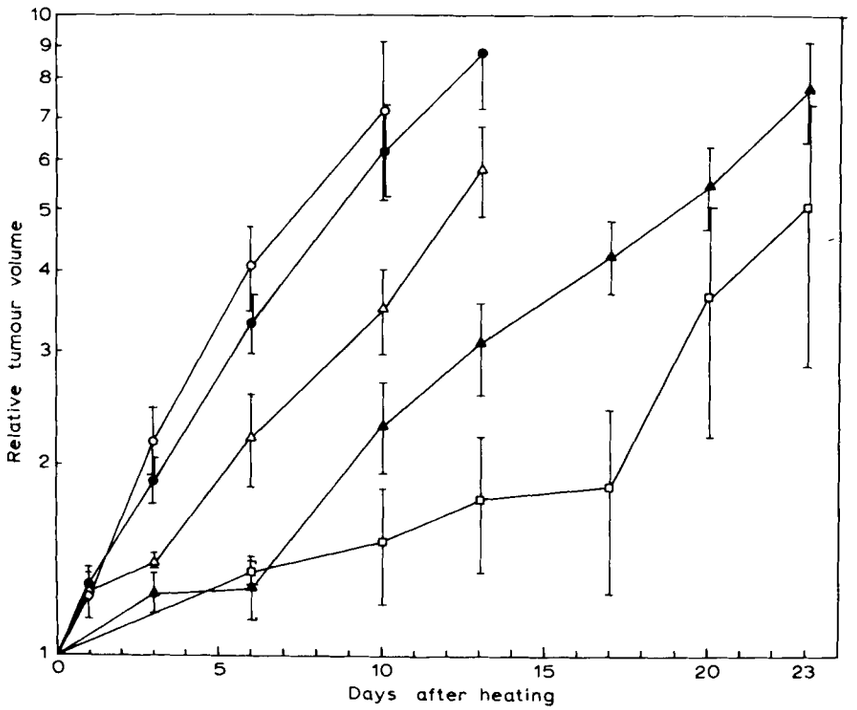

Fig. 5. Growth curves for solid BT_4A tumours treated by immersion of tumour-bearing legs in a water bath at 44.0°C for different times. Control, ambient temperature (○), 30 min (●), 60 min (△), 90 min (▲), 120 min (□). Each curve is the mean of groups of 9 to 11 rats. Vertical bars: ± SEM.

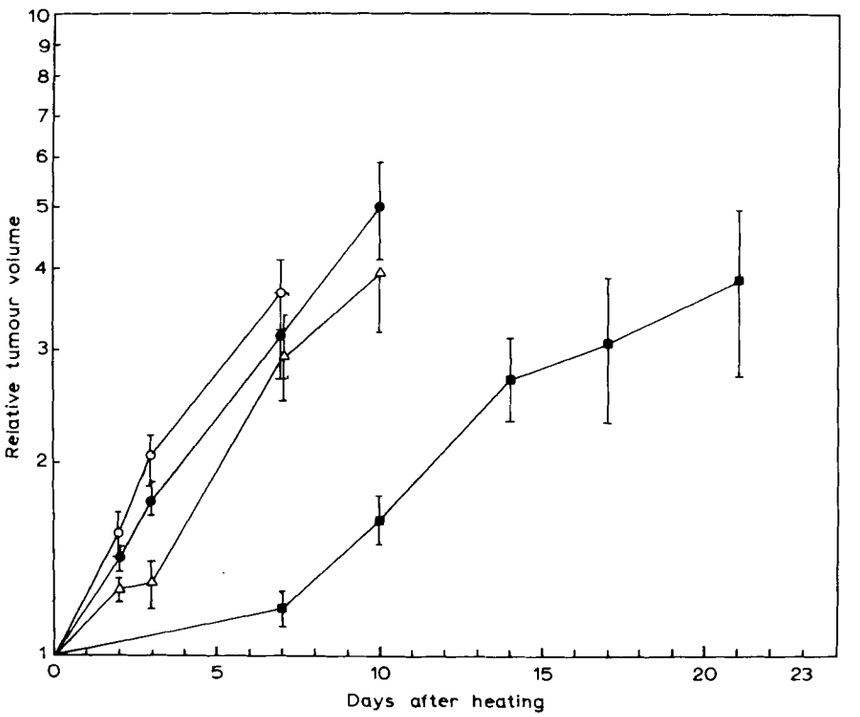

Fig. 6. Growth curves for solid BT_4A tumours treated by immersion of tumour-bearing legs in a water bath at 45.0°C for different times. Control, ambient temperature (○), 15 min (●), 30 min (△), 60 min (■). Each curve is the mean of groups of 9 or 10 rats. Vertical bars: ± SEM.

eter) were selected for the experiments. The rats were separated in groups according to the tumour volume and sex before randomization to treatment or control groups.

The tumour volume was calculated using the modified ellipsoid formula: $4/3\times\pi\times A/2\times(B/2)^2 = \pi/6\times A\times B^2$, where A is the longer axis and the perpendicular diameter B is assumed to be representative of the smallest semi-axis of the ellipsoid. Mean volume was 302 ± 15 (SEM) mm^3 on the treatment day.

Twenty-seven tumours were measured in vivo, then carefully excised and measured prepared free of connective tissue and the overlying skin. Using the formula mentioned, the ratio of volumes for the free-prepared tumours and the measurements in vivo was 1.01 ± 0.14 (SD).

Each tumour volume was normalized to one at the time of treatment. The growth curves are composed of two separate experiments using different passages. The tumours were assumed to have linear growth between the days of measurements (3–4 days) when calculating the tumour doubling time, TD. No significant change (F test) in the TD of the control groups occurred. Growth delay, GD, an estimate of the number of volume doubling times saved by hyperthermic treatment, was defined as described by NOWAK et coll. (1978): $GD = ({}^{TD}\text{treated} - {}^{TD}\text{control})/{}^{TD}\text{control}$. The animals were killed when the tumours became large and ulcerating (mean 10 times the initial volume). No metastasis was observed at section.

Side effects. The normal tissue reaction caused by hyperthermia was registered as edema lasting more than 48 hours after treatment. This edema was transient, resulting either in complete normalization within a few days or development of black skin that later formed crusts which healed with a scar or formed an ulceration. The skin damage was always seen immediately above the most prominent part of the tumours. More serious side effects like loss of toes, hemorrhage in the tumour area and even death occurred only in a few animals.

Statistical analysis. Data are given with standard deviation (SD) or standard error of the mean (SEM). The one-way analysis of variance (F test for equality of several independent means) and unpaired Student's t-test were used as described by IPSEN & FEIGL (1970). p-values under 0.05 were considered statistically significant. For statistical analysis of the correlation coefficient, r, calculated by linear regression, the formula $t=r\sqrt{(n-2)/(1-r^2)}$ was used at $n-2$ degrees of freedom.

Results

The tumours typically responded to hyperthermia by a temporary arrest of the tumour growth for shorter of longer periods of time. Thereafter, regrowth started and proceeded at approximately the same rate as before treatment when passing 200 per cent of their pretreatment volume, analysed by linear regression. No cure was achieved.

The growth curves for temperatures 42.0 to 45.0°C are shown in Figs 3 to 6. A slight but probably insignificant growth delay occurred after exposure for 2, 6 and 12 hours at 42.0°C compared with the control group. At 43.0 to 45.0°C a greater delay in growth was found with increasing treatment time above a threshold of 30 min.

TD and GD for each treatment group are given in Table 1. No significant increase of TD was observed at 2 to 12 hours at 42.0°C. At 43.0 and 44.0°C a graded increase of TD and GD was found after exposure times of 60, 90 and 120 min. At 45.0°C no significant TD increase was found below 60 min ($p=0.07$ at 30 min). GD above 2 was only found at the longer exposure times at 43.0 to 45.0°C.

Hyperthermic effect in vitro and in vivo. GD for transplantable tumours was correlated with the surviving fraction (SF) for the same cell line exposed to the same temperature and time in culture (DAHL 1980). The data showed a linear relation in a semilogarithmic plot (Fig. 7) where the abscissa is GD in vivo and the ordinate the logarithm of SF in culture: $y=-0.620\times-0.373$ (correlation coefficient: $r=0.94$, $t=8.44$, $n=12$, $p<0.01$). This means that the results found in culture predicted the thermal sensitivity found in vivo. One log reduction of SF corresponded to an increase of GD of one.

Side effects. The rats generally tolerated local hyperthermia very well except in the group exposed to 43.0°C for longer times, where one rat succumbed after 2.5 h exposure and one died 3 days after 4 h treatment. No weight loss occurred in any group measured one week after therapy.

Heated tumours and to a lesser degree control tumours appeared enlarged and red immediately following treatment in the water bath. Most tumours treated above 43.0°C looked cyanotic. The observed edema was rapidly reduced in many animals. Edema lasting more than 48 h after exposure is reported in

Table 1

Tumour doubling time (TD) and growth delay ($GD=(TD_{treated} TD_{control}/TD_{control})$ for solid BT_4A tumours treated by immersion of tumour-bearing legs in water bath at different times at 42.0 to 45.0°C. Statistical analysis: Unpaired Student's t-test. NS: Not significant differences ($p>0.05$)

Temperature (°C)	Time (min)	No. of rats	Tumour doubling time (days) ± SEM	Level of significance	Growth delay
42.0	Control	11	5.40±1.22	–	0
	120	10	5.93±0.61	NS	0.10
	360	9	5.95±0.82	NS	0.10
	720	9	8.13±1.88	NS	0.51
43.0	Control	14	3.27±0.24	–	0
	30	10	3.02±0.19	NS	−0.08
	60	11	6.43±1.12	0.01>p>0.001	0.97
	90	10	7.85±1.35	p<0.001	1.40
	120	13	9.95±1.54	p<0.001	2.04
44.0	Control	9	3.67±0.77	–	0
	30	11	3.88±0.71	NS	0.06
	60	11	6.41±0.72	0.05>p>0.02	0.75
	90	11	10.09±1.00	p<0.001	1.75
	120	9	17.06±2.17	p<0.001	3.65
45.0	Control	9	3.75±0.55	–	0
	15	9	5.32±1.07	NS	0.42
	30	9	6.17±1.07	NS (p=0.07)	0.65
	60	10	16.26±3.18	0.01>p>0.001	3.34

Table 2

Side effects after hyperthermia by immersion of the tumour-bearing legs in water bath at 42.0 to 45.0°C for different times. Skin damage was recorded as edema 48 h after treatment or development of crusts or ulcers (skin necrosis) the following week. Gross hemorrhage or loss of toes also occurred and 2 rats succumbed during or after treatment

Temperature (°C)	Time (min)	No. of rats	Edema 48 h after treatment	Crust or ulceration	Loss of toe, hemorrhage	Death
42.0	120	10	0	0	0	0
	360	9	0	0	0	0
	720	9	1	0	0	0
43.0	30	10	0	0	0	0
	60	11	1	0	0	0
	90	10	1	2	0	0
	120	13	2	3	1	0
	240	6	4	4	0	2
44.0	30	11	0	0	0	0
	60	11	4	1	0	0
	90	11	6	3	0	0
	120	9	9	9	5	0
45.0	15	9	1	0	0	0
	30	9	0	1	0	0
	60	10	8	7	0	0

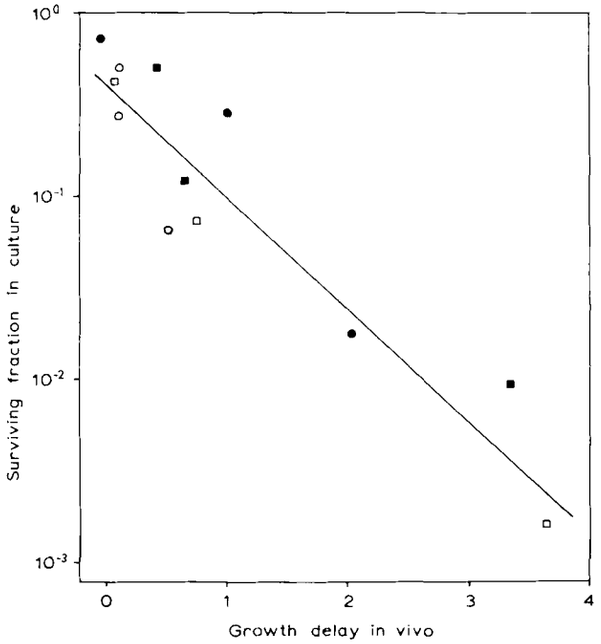

Fig. 7. The log-linear relationship between surviving fraction (SF) in vitro and growth delay (GD), doubling times saved by treatment, in vivo for BT_4 cells treated by hyperthermia. Each point is the result of the same exposure time in vitro and in vivo at 42.0°C (○), 43.0°C (●), 44.0°C (□), and 45.0°C (■). The formula of the line is $y = -0.620x - 0.373$, where x = GD and y = log SF. Three different times were measured at each temperature.

Table 3

Heating time for 50 per cent probability of induction of edema recorded after 48 h and skin necrosis recorded during the first week after hyperthermia

Temperature (°C)	Time (min) to induce	
	50 per cent edema	50 per cent skin necrosis
42.0	–	–
43.0	150	150
44.0	75	90
45.0	42	48

Table 2. After another one or two days this edema was reduced and no late skin reactions were seen at 42.0°C for exposure times below 12 h. At 43.0°C necrosis of skin which resulted in crusts on the tumours was found for heating times above 90 min. After 2 h, hemorrhage and loss of a toe occurred in one rat. At 44.0 and 45.0°C, crust formation and ulceration occurred after exposure times of 60 and 30 min, respectively. Two hours at 44.0°C resulted in gross normal tissue damage in 5 of 9 animals. The 50 per cent probability for development of edema and skin necrosis is presented in Table 3.

Discussion

The present results show that hyperthermia alone induced a progressive retardation in growth of the neurogenic rat cell line BT_4A with increasing treatment times at temperatures of 43.0 to 45.0°C. Similar results with no cures and regrowth after a delay are reported for several other cell lines after single treatments in vivo (glioma 26: MAGIN & JOHNSON; JOHNSON; B16 melanoma: WAHL et coll. 1979; human carcinoma: KIM & HAHN 1979) while other more sensitive cell lines can be cured at the same temperature and time combinations (mouse melanoma: CRILE 1961; anaplastic mouse mammary carcinoma: OVERGAARD & OVERGAARD 1972; Yoshida sarcoma in the rat: CALDERWOOD & DICKSON 1980; mouse fibrosarcoma: OVERGAARD & SUIT 1979, URANO et coll. 1980).

Heating technique. The observed differences in cure rates for different cell lines may be due to differences in intrinsic heat sensitivity. Several other factors may also influence the results.

The gradients between the water bath temperature and the recorded intratumoural temperatures are reported to be 0.2 to 0.3°C for some mouse tumours (STEWART & DENEKAMP 1978, OVERGAARD & SUIT, ALBERTS et coll. 1980, OVERGAARD 1980) in contrast to reports of thermal gradients of 0.5 to 1.7°C (DICKSON & ELLIS 1974, BLEEHEN et coll. 1977, ROBINSON et coll. 1978, HILL et coll. 1980, HUME et coll. 1980, WALLEN et coll.) for other rodent tumours. In the present experiments the thermal gradients were in the order of 0.2 to 0.3°C at 42.0 and 43.0°C, while the temperature first approached 44.0 and 45.0°C, then decreased after a few min and was then stable 0.5 to 0.8°C below the water bath temperature. A similar decrease in temperature is reported in normal dog muscle as the result of a physiologic heat adaptation by reflex vascular cooling (STORM et coll. 1979). This thermoregulating mechanism is inhibited by vascular clamping. Therefore, the present measurements indicate that the fixation by tape did not grossly obstruct the blood flow during hyperthermia.

In one tumour system (VAN DIJK & BREUR 1980) radiofrequency (RF) heating at 44.0°C for 50 min cured 50 per cent of tumours while 60 min at 44.5°C in a water bath only resulted in growth delay for 8 days and no cures. This may simply reflect a thermal gradient between tumour and water bath greater than 0.5°C. Non-thermal effects of microwaves (SZMIGIELSKI & BIELEC 1975) and ultrasound

(DUNN & POND 1978, LEHMANN et coll. 1978, TER HAAR et coll. 1980) might also contribute to the observed difference, but such effects are debated (LELE 1978). The insertion of probes for recording intratumoural temperature during RF heating might also influence the cure rate (HONESS et coll. 1978).

Tumour site. Subcutaneously implanted flank tumours grew faster when localized in the anterior part of the trunk of mice than in the posterior part (AUERBACH et coll. 1978 a, b, KYRIAZIS & KYRIAZIS 1980). This regional difference was also found by intraperitoneal injections in mice (MORRISSEY et coll. 1980). A similar difference was demonstrated for the neurogenic rat cell line BT_2A between the neck and thigh. Tumour growth was also slower in the dorsal part of the foot than in the thigh. The factors leading to the observed regional differences in growth have been attributed to persistence into adult life of anteroposterior gradients operating during ontogenesis (AUERBACH et coll. 1978 b), increased vascular supply in the cephalic end (KYRIAZIS & KYRIAZIS), regional differences in body temperature (AUERBACH, cited by KUMAR 1980) or increased skin organelles and nerve supply (AUERBACH et coll. 1978 a). The tumour growth may also be influenced by tissue pressure during expansion at the site of inoculation.

The pig skin was not uniformly susceptible to thermal injury (MORITZ & HENRIQUES 1947). The ears, thighs, buttocks and ventral surface were more and that of the neck and midportion of the back less vulnerable. Thus the site of inoculation will influence the tumour growth as well as the side effects.

Tumour volume. Larger tumours are reported to be more heat sensitive (CRILE 1963, STORM et coll., URANO et coll.), while DICKSON & ELLIS (1976) and CALDERWOOD & DICKSON reported that the larger ones were less heat sensitive. Differences in blood flow, microenvironment (pH, nutrition) as well as growth fractions for the tumour system used may explain the opposite conclusions.

Host factors. Hyperthermia as used in the present experiments did not induce starvation and weight loss, which have a known slowing effect on tumour growth (ROUS 1914, MULDER & VAN PUTTEN 1979).

Most murine tumours responding successfully to hyperthermia have been more or less immunogenic (MONDOVI et coll. 1972, SUIT 1975, MARMOR et coll. 1977, SHAH & DICKSON 1978, TWENTYMAN et coll. 1978, ALFIERI et coll. 1980). The lack of cure of the BT_4A tumours may be caused by a lack of host rejection of surviving cells in the inbred BD IX rat strain used.

Side effects. Generally the heating time must be reduced by a factor of 2 for a 1°C rise in temperature for skin damage in different species (MORITZ & HENRIQUES, CRILE 1961, LAW et coll. 1978, MORRIS et coll. 1978, OKUMARA & REINHOLD 1978), although the absolute time at a particular temperature varies (FIELD 1978). Corresponding to this general rule a factor of 1.8 for both edema and skin necrosis was calculated from Table 3.

Heat sensitivity in vitro and in vivo. The log-linear relation between SF and GD confirms previous results (WESTERMARK 1927, DICKSON & SUZANGAR 1974) where heat sensitivity in vitro was found to be a reliable guide to heat sensitivity in vivo. No cure was achieved at any of the time and temperature combinations used in the present experiments. As extremely low SF levels (less than 10^{-6}–10^{-7}; SUIT, URANO et coll.) probably are necessary to obtain tumour cure, the present in vivo results reflect similar heat sensitivity in vitro and in vivo as reported by MARMOR et coll. The reported greater (OVERGAARD 1977, KANG et coll. 1980) or decreased (WESTERMARK, JOHNSON 1940) heat sensitivity in vivo may be related to the tumour used, heating technique, and local factors such as pH, nutrition or hypoxia (OVERGAARD 1978).

In conclusion: The present experiments indicate that the transplantation of neurogenic tumours to hind limbs of rats can be used as a stable and standardized system for the investigation of local hyperthermic treatment. However, with the type of tumour employed, a single hyperthermic treatment is not sufficient for cure, showing that combination of either multiple treatments, cytostatic drugs or irradiation are necessary for further improvements. Such investigations are now in progress.

SUMMARY

The effect of hyperthermia alone on the growth of the BT_4A neurogenic tumour implanted into the feet of BD IX rats has been investigated. Following treatment by immersion of the tumour-bearing leg in a water bath at 42.0 to 45.0°C a temporary retardation of tumour growth was observed but no cure. The lag phase before regrowth occurred was temperature and time dependent. A log-liner correlation was found between the surviving fraction previously found in vitro and the heat sensitivity of the cell line in vivo.

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