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EFFECT OF HYPERTHERMIA ALONE AND IN COMBINATION WITH ^{60}Co RADIATION ON THE GROWTH OF B16 MELANOMA IN MICE

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For more than a hundred years heat has been assumed to have a destructive effect on tumour cells. Sporadic attempts at heat treatment on patients with malignant diseases have been performed, utilizing a variety of methods, causing tumour regression and even cures (CAVALIERE et coll. 1967, STEHLIN et coll. 1975).

During the last decades hyperthermia has gained additional interest, and many experiments have been performed to elucidate the influence of heat treatment on cells. An apparently selective heat sensitivity of malignant cells in vitro has been reported by many authors (CHEN & HEIDELBERGER 1969, GIOVANELLI et coll. 1973, GIOVANELLI et coll. 1976, KASE & KAHN 1976), while others propose that this is only a result of higher proliferative activity of malignant than of normal cells (DIETZEL 1975). A selective destruction of tumour tissue after hyperthermia treatment of several experimental animal tumours has been reported (CRILE 1963, DICKSON & ELLIS 1976, OVERGAARD & OVERGAARD 1972, 1976). According to Overgaard (OVERGAARD & OVERGAARD 1972, 1977) the reason for the selective heat sensitivity of malignant cells in vivo is the presence of more acidic conditions in the interior of a tumour. The difference in heat sensitivity between normal and malignant cells is small and appears in a narrow temperature range between 41°C and 43°C (GIOVANELLI

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1977, OVERGAARD & OVERGAARD 1977), but it makes hyperthermia an extremely interesting subject for further research in therapy of malignant tumours.

Hyperthermia has its greatest killing effect on cells in the S-phase of the cell cycle, which usually is the most radiation resistant phase, while the more sensitive G_1-phase is the most heat-resistant (KIM et coll. 1976, WESTRA & DEWEY 1971).

Radiation resistant hypoxic cells in certain tumours are at least as heat sensitive as well oxygenated cells (GERWECK et coll. 1974), and heat combined with irradiation has been shown to reduce the oxygen effect considerably (KIM et coll. 1975). Thus, hyperthermia has been proposed as a possible means of overcoming problems ascribed to radiation resistant hypoxic tumour cells, and might become an alternative to, for instance, high LET radiation, or hyperbaric oxygen therapy with low LET radiation.

When hyperthermia is combined with ionizing radiation, the lethal effect is enhanced (OVERGAARD & OVERGAARD 1974). It has been proposed that hyperthermia somehow interferes with the enzymatic repair of sublethal roentgen ray injury (BEN-HUR et coll. 1974). When the dose rate is reduced from values above 1 Gy/min, normally used in radiation therapy, to dose rates in the range 10^{-2} Gy/min, the cell survival has been shown to increase (HALL 1973), an effect ascribed to repair during the exposure. If this repair is inhibited, for instance by hyperthermia, the cell-killing increases. At a dose rate of 3.3×10^{-2} Gy/min, BEN-HUR et coll. found in in vitro experiments with hamster cells a 6-fold increase in sensitivity as the temperature during irradiation was raised from 37°C to 42°C.

A negative consequence of hyperthermia is a possible stimulation of metastatic spread of tumour cells. DICKSON & ELLIS (1974) treated Yoshida sarcoma, implanted in the leg of rats, with local hyperthermia (42°C for 1 h), a treatment which per se was inadequate for tumour destruction. Body temperature rose to 41.5°C during the treatment. The treatment was shown to result in a significantly enhanced metastatic spread of tumour cells to the regional lymph nodes and viscera as compared to untreated tumours. YERUSHALMI (1976) treated Lewis lung carcinoma, growing in the hind leg of mice, with local or whole-body hot air hyperthermia. Compared to untreated controls, the appearance of lung metastases was advanced in whole-body treated, and delayed in locally treated animals. One explanation is that increased temperature stimulates premature activity of tumour cells present at the metastatic site (DICKSON & ELLIS 1974); another is that increased body temperature causes a depression of the immune defence mechanism of the host, a view which is supported by HARRIS (1976), who found that cytolytic activity of T-lymphocytes in culture was decreased by more than 99 per cent after exposure to 42°C for 45 min. Thus, it seems that increased body temperature may stimulate metastatic spread of tumour cells. If this proves to be a general result, local hyperthermia is to be preferred to whole-body hyperthermia in the treatment of malignant tumours.

At present no agreement exists as regards what sequence of hyperthermia and irradiation is the most efficient, but DEWEY (1977) reported in a review article that

irradiation followed by hyperthermia tends to be more effective at low temperature hyperthermia (i.e. temperatures below 43°C), while the opposite seems to be true for higher temperatures.

A temperature of 41.5°C is well tolerated by patients, even whole-body treatment for several hours (PETTIGREW et coll. 1974) and is thus of clinical relevance. This temperature was therefore chosen in the present work, applied locally and, in experiments with high dose rate irradiation, during and after irradiation. Mice were used as test animals. The tail was chosen as tumour site because it is well suited for local heat treatment as well as for local irradiation.

Human melanomas often have a poor response to irradiation and chemotherapy. In the present work the combined effect of hyperthermia and irradiation on a murine melanoma was therefore investigated to shed light on whether hyperthermia may become a means to improve the treatment of melanomas.

Materials and Methods

B6D2F1/BOM mice of both sexes 7 to 8 weeks old were used in the experiments. The tumour, the murine B16 melanoma, was maintained by repeated passages in the right thigh of animals every second week. Cell inocula were prepared by removing cells from a tumour, followed by filtering through a fine stainless steel mesh, and mixing with saline 0.9 % up to three times the cell volume. Aliquots of 0.1 ml of this cell suspension were in some of the experiments injected intramuscularly in the right thigh with a 21G needle. Tumours became visible within 7 days, grew quickly and reached a diameter of 20 mm after 3 weeks. The mice died about 3 weeks after injection. For most experiments, approximately 0.02 ml cell suspension was injected intramuscularly in the tail of the mice. Tail tumours grew more slowly than tumours in the thigh. They became visible 2 to 3 weeks after injection, and reached a suitable volume for treatment 3 to 4 weeks after injection. Mice with tail tumours died 5 to 7 weeks after injection.

The tumours were measured at least three times a week with a slide gauge. Tumours were assumed to be an ellipsoid, and the volumes were calculated from the formula $4\pi/3(a \cdot b \cdot c)$, where a, b and c are the three semi-axes of the ellipsoid. The volume of the tail extending through the tumour was not subtracted; this represents, however, a small error, as tumour to some extent infiltrated the tail. The assumption that tumour is an ellipsoid is obviously not always correct, and this may cause some error. The total tumour volume varied considerably from animal to animal in each group. The length of the tumour (2c) proved to be particularly variable from the very start of treatment. Each tumour volume was normalized to unity at the time of treatment.

In each experiment tumour sizes were measured every 2 to 3 days, and the volumes on the days between measurements were estimated by linear interpolation. Each

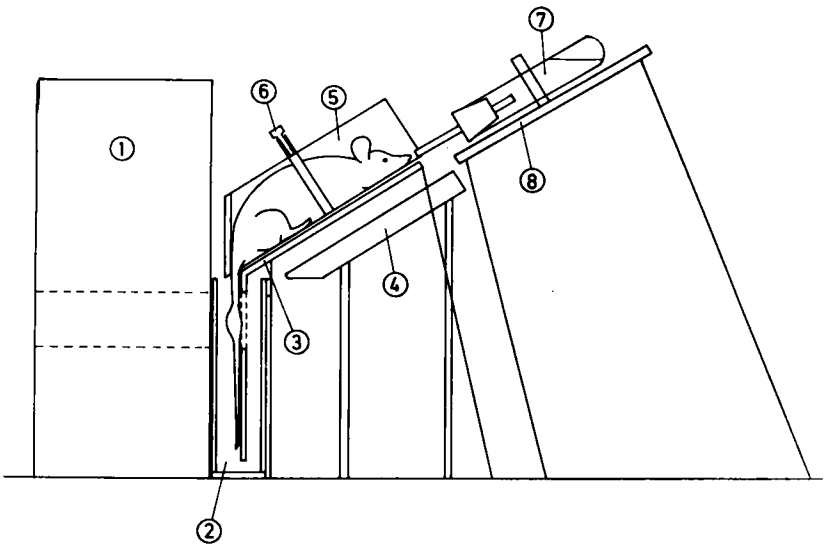

Fig. 1. Experimental set-up for irradiation of tumours in the tail of mice. The mouse is placed in a perspex jig (5) which is fastened to the stand (3) with a screw (6). The tail is immobilized with tape and placed in the water tank (2) with the tumour positioned in the middle of the radiation field behind the collimator (1). A brass plate (4) protects the animals from scattered radiation. The mice receive water during irradiation from a tube (7) which is fastened to a stand (8).

growth curve has been based on several identical experiments. Mean tumour volumes were calculated as the average value of the tumour sizes of all individual mice in each group.

In each experiment a group of untreated mice served as controls. For each treatment modality the mean tumour volumes of the corresponding controls were calculated in the same way as that for treated animals.

Irradiation was carried out with a ^{60}Co therapy unit, TEM Mobaltron 80. The mice were positioned as shown in Fig. 1. To obtain appropriate dosimetric conditions, the mouse tails were irradiated in a water phantom, e.g. a specially designed perspex water tank 35 cm × 9 cm × 24 cm (width, height and depth, respectively), the wall thickness of which was 2 mm. The distance from the outer surface of the water tank to the tumour center was approximately 12 mm, which is beyond the maximum of the depth dose curve of ^{60}Co radiation. The mice were put in perspex jigs supplied with many air-holes and immobilized with a piston. The jigs were fastened to a stand and the tails placed in the water tank, with the tumour in the middle of the radiation field. The experiments were carried out at two different dose rates:

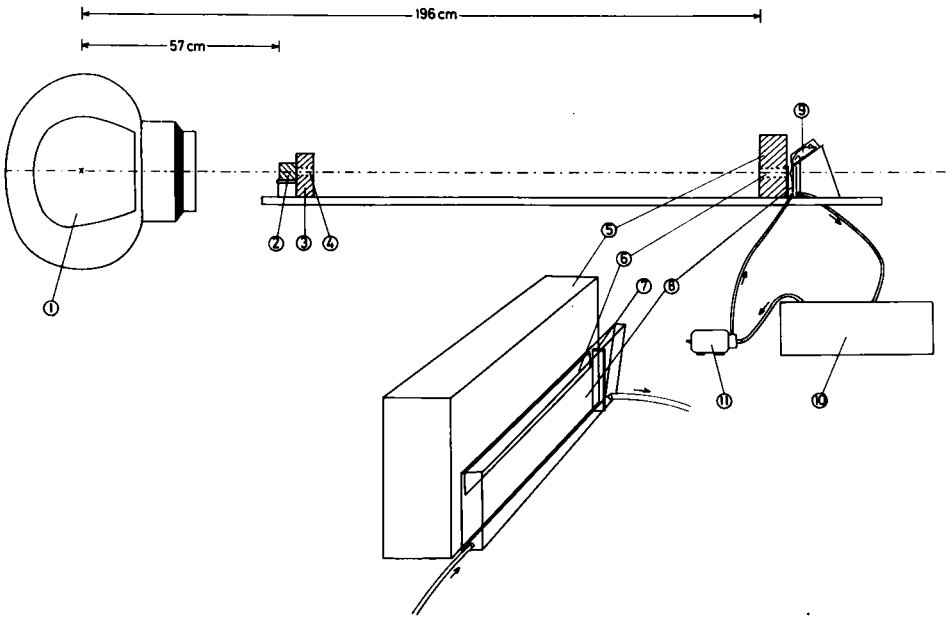

Fig. 2. Experimental set-up for simultaneous heating and irradiation of tail tumours at low dose-rate. ^{60}Co radiation from a Mobaltron 80 (1), placed horizontally, passes through a 5 cm thick brass filter (2), a 5 cm thick lead collimator (3) with aperture 12 cm × 2 cm (4), and an 8 cm thick lead collimator (5) with aperture 35 cm × 2.5 cm (6). The collimators are mounted on a transportable table. The mouse (9) is placed with the tail in the water tank (8) as shown in detail in Fig. 1. Hot water from the water bath (10) is pumped (11) up to the water tank and returns to the water bath over a threshold (7). Eight animals can be treated simultaneously.

The set-up for experiments with reduced dose rate is shown in Fig. 2. The distance between source and water tank surface was kept at 204 cm and a 5 cm thick brass filter was placed in the beam 57 cm away from the source. To obtain a rectangular and homogeneous field, the beam passed through two lead collimators, the first placed 62 cm away from the source, 5 cm thick with aperture 12 cm × 2 cm, the second 8 cm thick with aperture 35 cm × 2.5 cm, placed 196 cm away from the source. The dose rate at the tumour position, as measured by an ionizing chamber, was 2.0×10^{-2} Gy/min. Eight animals could be treated simultaneously.

For conventional dose rate experiments, the distance between source and water tank surface was kept at 80 cm. An 8.5 cm thick lead collimator, placed in front of the water tank, reduced the field size to 2.5 cm × 2.5 cm. The dose rate at the tumour position was 1.29 Gy/min. In this experimental set-up only one animal could be treated at a time.

Heating. Hyperthermic conditions were obtained by circulating hot water through the water tank, as demonstrated in Fig. 2. Hot water from a thermostatically con-

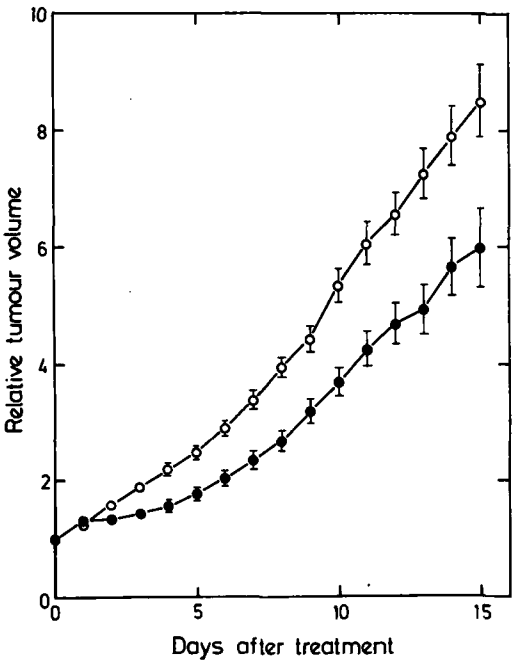

Fig. 3. Tumour growth following heat treatment at 41.5°C for 200 min performed on day 0. ● = treated tumour (37 animals), ○ = untreated tumour (44 animals). Bars indicate standard error of the mean.

trolled water bath (Heto, model 01T, Denmark), the temperature of which was kept constant within ±0.1°C, was pumped (Charles Austen Pumps Ltd., type C16/300, England) through plastic tubes up to the water tank. The water ran via a threshold back to the water bath. Water bath temperature and pump speed were adjusted so as to achieve the desired temperature (within ±0.2°C) in the water tank.

The exposure time for a total dose of 4 Gy, delivered at a low dose-rate (2×10^{-2} Gy/min), was 200 min, and heat treatment was, in this case, maintained without interruption during irradiation.

The exposure time for the same dose, delivered at conventional dose-rate, was approximately 3 min. Immediately after irradiation under these conditions, the mice were transferred to a water bath outside the irradiation room for the remaining part of the 200 min hyperthermic treatment period, a process which interrupted the heat treatment for about 3 min.

Intratumour temperature was measured with a needle thermocouple probe (diameter 0.7 mm) connected to an electric universal thermometer (Ellab A/S, type TE 3-S, Denmark). The temperature in the tail and in the tumour was found to lie within the range 20°C to 32°C when the mice were kept in room temperature. When the tail was put in hot water, intratumour temperature rose to that of the water within 2 min. Intratumour temperature was about 34°C for tumours growing in the thigh of the animals. When the leg was put in hot water, intratumour temperature rose

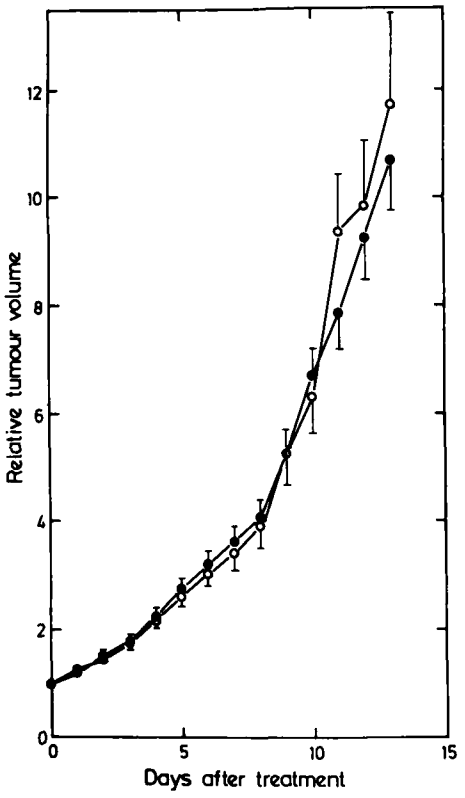

Fig. 4. Tumour growth following a single dose of 4 Gy, given on day 0 at 30°C and dose-rate 1.29 Gy/min. ● = treated tumour (18 animals), ○ = untreated tumour (23 animals). Bars indicate standard error of the mean.

to that of the water within 5 min. In both cases the deviation between intratumour and water temperature was less than 0.1°C after equilibrium was reached.

Results

Hyperthermia alone. Hyperthermic treatment at 41.5°C for 200 min caused a delay of tumour growth compared to that of untreated controls (Fig. 3). On the first day following the heat treatment, the tumours were red, soft and somewhat swollen. During the following days the tumours gradually became darker and harder, and growth was delayed for 2 to 3 days. Thereafter treated tumours grew about as quickly as untreated tumours.

Heat treatment at 43°C for 60 min caused approximately the same growth delay as 41.5°C for 200 min. These results agree well with those reported by SUIT & SHWAYDER (1974), who, on the basis of several reports, found that an increase in temperature by 1°C reduces the treatment time necessary for the same effect by a factor of 2.

In another preliminary experiment tumours were treated at 43°C for 200 min.

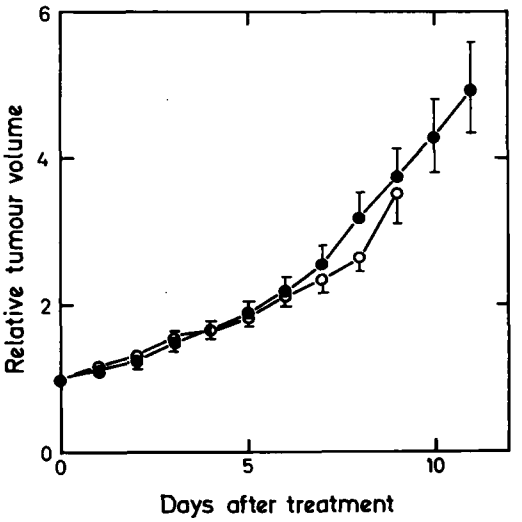

Fig. 5. Tumour growth following a single dose of 4 Gy, given on day 0 at 30°C and with a dose-rate 2.0×10^{-2} Gy/min. ● = treated tumour (10 animals), ○ = untreated tumour (13 animals). Bars indicate standard error of the mean.

This treatment lead to tail rupture at the tumour site, but regrowth of the melanoma appeared at the end of the ruptured tail. It therefore seems to be impossible to cure the B16 melanoma in the tail of mice with a single heat dose alone.

Irradiation at 'normal' tail temperature. In order to determine the radioresponse of the B16 melanoma in the tail of mice at or near the normal tail temperature, irradiation was performed with the tails placed in water kept at 30°C. Figs 4 and 5 demonstrate that a single dose of 4 Gy had no effect on tumour growth, either at conventional dose rate, or at a low dose rate. Preliminary experiments showed furthermore that doses of less than 15 Gy caused an insignificant delay in tumour growth. Doses higher than 15 Gy reduced the tumour volume somewhat, but regrowth started about a week after irradiation.

Simultaneous hyperthermia and irradiation. The tails were put in the water tank about 5 min before irradiation to permit the tumours to reach the desired temperature.

The growth curves (Figs 6 and 7) show that a dose of 4 Gy combined with hyperthermia had a marked and significant effect on tumour growth, both when hyperthermia was combined with low dose rate irradiation (Fig. 7) and with conventional dose rate irradiation (Fig. 6). Growth in both cases was inhibited for 3 to 4 days, and the tumours became darker and harder. Thereafter regrowth started, and cures never occurred. The inhibiting effect on tumour growth of a dose of 4 Gy, delivered at low dose rate, appears to be somewhat greater than that of the same dose at high dose rate, but the difference is small and not significant. The normal part of the tail

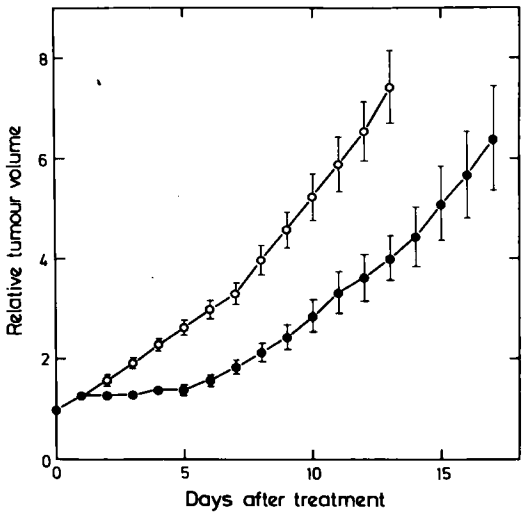

Fig. 6. Tumour growth following a single dose of 4 Gy, given on day 0 at dose-rate 1.29 Gy/min, combined with hyperthermia (41.5°C for 200 min) during and after irradiation. ● = treated tumour (24 animals), ○ = untreated tumour (34 animals). Bars indicate standard error of the mean.

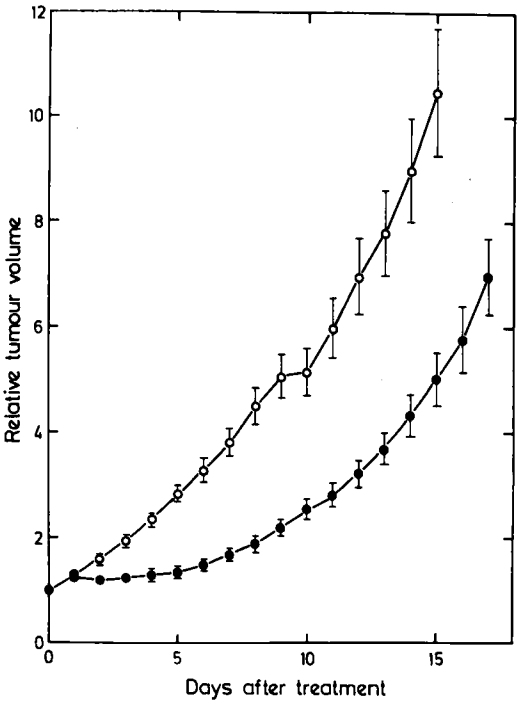

Fig. 7. Tumour growth following a single dose of 4 Gy given on day 0 at dose-rate 2.0×10^{-2} Gy/min, combined with hyperthermia (41.5°C for 200 min) during irradiation. ● = treated tumour (35 animals), ○ = untreated tumour (30 animals). Bars indicate standard error of the mean.

Table

Growth delay of treated tumours to attain the relative volume 2

Temperature (C)/ time (min)	Dose (Gy)	Dose rate (Gy/min)	Growth delay (days)
30°/3	4	1.29	0
30°/3	4	0.02	0
41.5°/200	0		2.5
41.5°/125*	2.5	0.02	3
41.5°/200	4	1.29	4
41.5°/200	4	0.02	5
41.5°/300**	6	1.29	7
41.5°/300**	6	0.02	6

* Result from 5 treated and 3 untreated tumours
** Result from 6 treated and 9 untreated tumours
Remaining data compiled from experiments referred to in the text.

tolerated these combined treatments well; only the tumours seemed to be affected. A dose of 6 Gy given at low dose rate combined with hyperthermia (41.5°C for 300 min) had a greater growth-reducing effect, but many tails were lost.

Effect of hyperthermia on metastatic spread of tumour cells. When untreated tumour-bearing animals were killed and dissected three weeks after injection, melanoma cells were found in the lungs of 50 to 80 per cent of the animals. These appeared as black spots in the lung tissue, and were confirmed at microscopy. This high incidence of tumour cells in the lungs was probably partly due to cells being injected directly into the veins of the tail. Thus, tail tumours were considered unsuited for detecting whether heat treatment increases the frequency of lung metastases.

When tumours were implanted in the thigh of the animals, melanoma cells seldom were found to spread to the lungs. Therefore tumours implanted in the thigh were used to investigate whether local heat treatment influenced the spread of tumour cells.

The mouse was placed in a mouse jig with the tumour-bearing leg protruding through a hole in the wall of the jig and fastened with tape. The jig was positioned so that only the tumour-bearing leg was immersed in water. Several holes were drilled in the jig, and a hair-dryer blew air of room temperature on the mice to reduce the heating of the animals by steam from the water bath.

Rectum temperature was found to be 36°C to 37°C when the animals were kept at room temperature, but it rose to 38°C to 39°C when the thigh was heated at 41.5°C, and to 39°C to 40°C when heated at 43°C.

Mice were heat-treated 10 days after injection of melanoma cells, at which time the tumour volume was about 0.5 cm^3, and killed 7 days after the heat treatment.

Heat treatment of the thigh at 41.5°C caused, as mentioned, about 2 degrees rise in rectum temperature. The animals did not tolerate this treatment for 200 min, 50 per cent died during or just after treatment. Heating at 41.5°C for 120 min was tolerated, as was heating at 43°C for 30 min, which caused a rise in rectum temperature of about 3 degrees.

Lung metastasizing frequency of mice receiving heat treatment was one out of 10 mice treated with 41.5°C/120 min and 3 out of 17 among the untreated mice. At 43°C/30 min one out of 8 treated and 2 out of 8 untreated mice developed metastases. Neither treatment regimen caused any significant change in the incidence of metastases compared to that of untreated tumour-bearing controls.

Discussion

The effect of a certain treatment was measured as tumour growth delay, i.e. the time that elapses from the group of control tumours have reached the relative volume 2 until the group of treated tumours reach the same volume. The results are summarized in the Table. Growth delay increases with increasing dose and increasing duration of the heat treatment. A dose of 4 Gy under normal body temperature has no effect on tumour growth, while the same dose, combined with hyperthermic treatment at 41.5°C for 200 min, has a considerable growth-inhibiting effect. Therefore it seems that the combination of a small radiation dose and a moderate hyperthermic treatment has a greater killing effect on B16 melanoma cells than either of the two treatments alone.

No significant difference was found between the regrowth delay of radiation doses of 4 Gy and 6 Gy delivered at high dose rate at the beginning of a heat treatment period of 200 min, and that caused by low dose rate irradiation during the entire hyperthermia period. This appears to be in contrast with the great cell killing effect observed under in vitro conditions, caused by irradiation at low dose rate combined with simultaneous hyperthermia, as reported by BEN-HUR et coll. (1974). Thus, the present experiments indicate that the restitution processes of B16 melanomas during low dose rate irradiation and those following a short, high dose rate irradiation, are inhibited nearly to the same extent by hyperthermia.

A moderate local hyperthermic treatment, e.g. 41.5°C for 2 hours, did not enhance the metastatic spread of melanoma cells to the lungs. This treatment seems, in this regard, to be no hazard to the animal.

The standard errors of the mean in Figs 3 to 7 appear to be relatively great. This may in part be due to small variations in temperature and perhaps to variations in blood supply to tumour. In addition, since the tail contains little muscle tissue, injection is difficult, and there may be some variations in the volume of cell inocula. Cell concentration in the inocula was not counted, and may have varied somewhat from group to group.

If normal tissue is sensitized to the same degree by hyperthermia as is tumour

tissue, the combined treatment would be of no advantage in the treatment of malignant tumours. The response of normal tissue to combined treatment must therefore also be investigated. Experiments comparing the enhanced radiation response induced by hyperthermia of tumour and normal tissue have now been initiated.

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SUMMARY

B16 melanoma was grown in the tail of B6D2F1/BOM mice. Procedures for simultaneous local ^{60}Co irradiation and heat treatment are described. A dose of 4 Gy had no effect on tumour growth; heat treatment at 41.5°C for 200 min had a minor effect, while the combined treatment caused a marked delay in tumor growth. Heat treatment of tumours in the thigh at 41.5°C for 2 hours did not influence the frequency of lung metastases.

ZUSAMMENFASSUNG

B16 Melanom war im Schwanz der B6D2F1/BOM Mäuse implantiert. Methoden für simultane lokale ^{60}Co Bestrahlung und Wärmebehandlung werden beschrieben. Eine Dosis von 4 Gy hatte keine Wirkung auf die Tumorentwicklung, Wärmebehandlung bei 41,5°C während 200 Minuten hatte nur eine geringe Wirkung, während die Kombination von Wärme und Bestrahlung eine erhebliche Verspätung der Tumorentwicklung verursachte. Wärmebehandlung bei 41,5°C während 2 Stunden von Tumoren, die im Schenkel implantiert waren, änderte nicht die Häufigkeit der Lungenmetastasen.

RÉSUMÉ

Un mélanome B16 a été implanté dans la queue de souris B6D2F1/BOM. Les auteurs décrivent les techniques de traitement simultané par l'irradiation locale du ^{60}Co et par la chaleur. Une dose de 4 Gy n'a pas eu d'effet sur la croissance tumorale. Le traitement par la chaleur à 41,5°C pendant 200 minutes a eu un effet minime alors que les traitements combinés ont entrainé un retard marqué dans la croissance tumorale. Le traitement par la chaleur de tumeurs de la cuisse à 41,5°C pendant 2 heures n'a pas influencé la fréquence des métastases pulmonaires.

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