Effects of Hyperthermia on Primary and Metastatic Tumor Growth and Host Immune Response in Rats

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ABSTRACT

A hot water bath was used to heat locally a metastasizing carcinoma in Wistar/Furth rats. Applying heat such that intratumor temperature is maintained at a mean value of 42.3° for two 90-min sessions results in a decreased growth rate of the primary tumor as well as distant metastases. Heating the primary tumor for only one 90-min session or heating the leg contralateral to the tumor-bearing limb has no effect on the growth rate of either the primary tumor or metastases. Heat therapy has no detrimental effect on the spleen cell-mediated tumor immune response of rats as tested by an in vitro lymphocytotoxicity assay 1 day later. However, heating isolated spleen cells to similar temperatures in vitro reduces their capacity for in vitro tumor cell killing.

INTRODUCTION

For over a century it has been repeatedly reported that systemic or local application of heat may be of medical benefit in a variety of diseases, including cancer. The literature contains numerous reports of the arrest or disappearance of malignant tumors after prolonged fevers or of local heating, e.g., the report of Cavaliere et al. (2). Overgaard and Overgaard (13) have provided an extensive literature review of clinical and experimental evidence indicating that tumor cells may be selectively killed by high temperature.

It is generally acknowledged that cellular immune reactions can influence tumor growth but the available evidence on the effects of hyperthermia on host immune response is contradictory. Dickson (4) reported that hyperthermia in rats or rabbits sufficient to cause regression of a primary tumor is accompanied by enhanced metastases. A depression in the immune defense mechanisms of the host at increased body temperature is suggested as a possible cause. In a preliminary report, Schaeffer (14) noted that whole-body hyperthermia increases the spread of artificially induced metastases in mice. The view that heat lowers host immune response is supported by Harris (10), who reported that cytotoxic mouse spleen cells when heated in vitro at 43° for 45 min lost their capacity to kill specific tumor target cells.

In contrast to the above, there is clinical evidence consistent with hyperthermia stimulating patient antitumor immunity. In some instances of multiple tumor foci, heat treatment of 1 focus resulted in regression of both the treated and untreated tumors (3, 17). Goldenberg and Langner (9) investigated hamsters bearing bilateral tumors and reported impaired growth of untreated tumor following heat treatment of the contralateral tumor. Mondovi et al. (12) reported that Ehrlich ascites tumor cells killed by hyperthermia are more immunogenic than cells inactivated by X-irradiation. Stehlin et al. (17) reported that the in vitro cytotoxicity of patient lymphocytes toward their own tumor cells is enhanced after hyperthermia and therefore postulated that heat renders tumor cells more antigenic, stimulating antitumor immunity of the patient. Suit (16) however, in a preliminary report, found heated fibrosarcoma cells less immunogenic in mice than irradiated cells.

In this communication, using a rat metastasizing tumor, both local and distal progression of the disease in response to hyperthermia are correlated with the effects of the therapy on in vitro cell-mediated cytotoxicity, in an attempt to resolve some of the apparent contradictions relative to the effects of hyperthermia on the host immune response.

MATERIALS AND METHODS

Animals and Tumors. The mammary carcinoma, termed Me-H, originally arose spontaneously in a Wistar/Furth rat (Microbiological Associates, Bethesda, Md.) and gave occasional metastases in rats with transplants having large primaries to lymph nodes, pancreas, and lung. The tumor is maintained by i.m. transplantation of tumor homogenate to the hind leg. After several years of passaging in this syngeneic strain, the tumor developed the capacity to rapidly and aggressively metastasize to the retroperitoneal lymph nodes and lung. At its present stage of evolution, the tumor metastasizes early in 100% of the rats but only to the retroperitoneal lymph nodes. Multiple foci are not observed. An injection of 10⁵ cells gives rise to a barely palpable tumor within 7 to 9 days. At this stage there are no gross metastases but if the primary is excluded, a metastasis develops within 14 days to the same extent as in intact rats. If left untreated the tumor grows continuously and kills 100% of the rats in 4 to 5 weeks.

The Wistar/Furth rat fibrosarcoma BP-11 was originally induced by benzopyrene. The tumor is maintained by i.m. transplantation of tumor homogenate or cultured cells in syngeneic rats and is 100% transplantable and lethal in 6 to 8 weeks. An injection of 10⁸ cells gives rise to a barely palpable tumor in 10 to 12 days. The tumor is nonmetastasizing and can be controlled by surgical excision before it is more than 2 to 3 cm in diameter. The BP-11 tumor is not immunologically cross-reactive with the Me-H tumor.

Cell Culture. Explants of the Me-H and BP-11 tumor were grown in monolayer culture in Earle’s minimal essential medium containing 10% heat-inactivated fetal calf serum (Associated Biomedic Systems, Buffalo, N. Y.). Assays...
for Mycoplasma were negative.

Hyperthermia. Rats with a barely palpable tumor on their right hind leg were anesthetized with sodium pentobarbital (25 mg/kg) and placed on a plexiglass platform resting over a 42.6 to 42.8°C water bath. The temperature of the bath was controlled with a thermoregulator (Arthur H. Thomas Co., Philadelphia, Pa.) sensitive to ±0.1°C. The tumor-bearing leg was immersed in the water bath through a hole in the platform, so that the tumor was completely submerged. Temperature monitoring probes (Type 402; Yellow Springs Instrument Co., Yellow Springs, Ohio) were inserted rectally and the temperature was measured at 5-min intervals by means of a multiprobe 6-channel telemeterometer (Model 44TF; Yellow Springs Instrument Co.). The thermometer is accurate to ±0.12°C. The temperature was found to increase with increasing depth of insertion of the rectal temperature-monitoring probe to a maximum of about 9 cm past the anus in 200- to 250-g rats. At this depth the sensor is in the transverse colon and this is the position routinely used. Intratumor temperature was monitored with a 24-gauge hypodermic needle probe (Type 524; Yellow Springs Instrument Co.), positioned so that the temperature sensor was at about the center of the tumor mass. Immediately after hyperthermia, each rat was given an injection i.p. of 1.0 ml of a 37°C 0.9% NaCl solution to replace fluid loss. The animal was then placed in a 37°C hot-air oven for 30 min to allow body temperature to return to normal. Sham-heated rats were treated in the same manner except that their tumor-bearing leg was immersed in a 37°C water bath.

For in vitro heating of rat spleen cells, nonadherent cells were suspended in RPMI-1640 plus 10% FCS. A test tube containing the cell suspension was placed in a water bath at appropriate temperature for 1 hr.

Cell-mediated Cytotoxicity Assay. Assay is performed by a modification of the method of Bean et al. (1). The plates are incubated at 37°C in 5% CO₂ in air for 2 days and then washed 3 times with 37°C phosphate-buffered saline (0.01 M Na₂HPO₄·0.14 M NaCl, pH 7.2) containing 0.5% ovalbumin using a modification of the method of Seeger et al. (15). Plates are washed by immersion and gentle movement in the baths. The plates are inverted in the first but not the last 2 rinses to facilitate removal of nonadherent cells (lymphoid cells and nonviable tumor cells). After this, plates are given a final wash in 37°C phosphate-buffered saline without ovalbumin, allowed to dry upright, and the bottom of the wells containing labeled tumor cells are punched out and processed for liquid scintillation counting as previously described (1). For prelabeling of tumor target cells, subconfluent monolayer cultures in Falcon 3013 flasks (Falcon Plastics, Oxnard, Calif.) are washed 3 times with Earle's minimal essential medium (proline free) containing 10% FCS. Five ml of the same medium and 75 μCi of [³H]proline (24.5 Ci/m mole; New England Nuclear, Boston, Mass.) are added to the flasks, and the cultures are incubated 20 to 24 hr. After labeling, the cultures are washed twice with RPMI-1640 (containing unlabeled proline), with 10% FCS and detached with 0.2% trypsin. After 2 other washes with RPMI-1640 and 10% FCS, the cells are suspended in this same medium at a concentration of 3 × 10⁶ cells/ml. A 0.1-ml cell suspension (3000 cells) is seeded into the wells of Falcon 3040 microtiter plates. Prior to addition of effector cells, the labeled target cells are allowed to attach to the well bottom at 37°C in 5% CO₂ in air for 1 to 2 hr.

Effector cells are derived from the spleens of tumor-bearing rats. Freshly excised spleen is placed in RPMI-1640 plus 10% FCS and squeezed with forceps to release cells. The resulting cell suspension is allowed to stand for 2 to 3 min to allow clumps to settle. The supernatant fluid is then aspirated and subjected to RBC lysis by a modification of the method of Falk et al. (8), which consists of suspending cells in 1.0 ml of FCS. To this is added 5.0 ml of 0.83% NH₄Cl and 0.017 M Tris (pH 7.2) at 37°C. The cell suspension is allowed to incubate for 2 min, after which 9 ml of ice-cold RPMI-1640 plus 10% FCS are added. The cells are immediately pelleted by centrifugation at 100 x g for 5 min. After 1 wash with RPMI-1640 plus 10% FCS, macrophages are eliminated by allowing them to adhere to the bottom of a Falcon 3024 plastic flasks for 1 hr at 37°C, and purified lymphocytes are added to the wells of a microtiter plate already containing labeled target cells for an effector:target cell ratio of 200:1.

Statistical Analysis. The statistical significance of differences between mean cpm or mean tumor volume of 2 different groups of rats was determined by Student's t test.

RESULTS

Heating rats to a core temperature of 40.7°C for 1 hr was found to be the maximum consistent with survival of most of the animals. Of approximately 70 rats subjected to this regimen, 2 died within 1 day after treatment for reasons that could possibly be ascribed to hyperpyrexia. Maximum tolerable hyperthermia was routinely used.

About 30 min are required for intratumor temperature to rise to a maximum of 42.3 ± 0.1°C and about 60 min are required for rat core temperature to reach 40.7°C. Rats were maintained at a 40.7°C core temperature for 60 min and at a 42.3°C intratumor temperature for 90 min. Total exposure to the hot water bath was 120 min.

Four rats were treated simultaneously in the same water bath and there was some individual variation in core temperature, probably due to slight differences in tumor size and depth of leg immersion. In order to eliminate this as an experimental variable and maintain a constant core temperature of 40.7 ± 0.2°C, when the core temperature rose above 40.7°C, a block was placed under the rat's head and thorax to allow for greater radiational cooling. When core temperature dropped below 40.7°C, the rat was warmed by covering with a blanket.

Effects of Hyperthermia on Primary and Metastatic Tumor Growth. In Chart 1, the results of heating of the Me-H tumor-bearing or contralateral limb are compared in terms of the effect on growth of the primary tumor. A single session of hyperthermia to the ipsilateral leg has no significant effect, but 2 sessions on consecutive days result in significant retardation in the rate of tumor growth (p < 0.02 by Day 6; p < 0.003 by Day 8 after initiation of treatment) relative to sham-heated controls. The effect of
hyperthermia on tumor growth is transient and by 8 days after initiation of treatment, the rate of tumor growth in the heated rats parallels that of the controls. Neither 1 (data not shown) nor 2 sessions of hyperthermia to the contralateral leg has a significant effect on tumor growth.

The effects of heat treatment on metastatic growth of the tumor are summarized in Table 1. Two sessions of local hyperthermia to the tumor-bearing limb result in significant reduction in the weight of a retroperitoneal metastatic mass. The effect on tumor metastasis is not the result of the relatively mild whole-body hyperthermia attendant with local heating, as indicated by the fact that the reduction in the weight of metastases is not seen when the contralateral leg is heated. The effect on metastases is not secondary to destruction of primary tumor cells per se because amputation of the primary is not followed by reduction in metastatic weight.

**Histology of the Me-H Tumor.** Sections of the primary tumor and nodal metastases reveal that they both have a similar histological pattern, i.e., anaplastic carcinoma with nuclear atypia, multiple mitotic figures and with many nuclei containing several nucleoli. One day after hyperthermia treatment the tumor shows pyknosis, inflammation, and necrosis, indicating cellular injury.

**Effects of Hyperthermia on Cell-mediated Cytotoxicity.** Spleen cells from rats bearing barely palpable Me-H tumors were heated in vitro for 1 hr at various temperatures (Table 2). Immediately after heating, the cells were tested for their ability to induce detachment of tumor target cells. Heat caused a dramatic reduction in the cytotoxic capacity of spleen cells toward an immunologically specific target. The minimum temperature at which reduced cytotoxicity is observed is 40.5°C, which approximates the temperature adjacent to the rat spleen during the regimen of in vivo heat therapy sufficient to cause a reduction in the rate of tumor growth. Heating spleen cells above 40.5°C results in further reduction in their cytotoxic capacity but does not affect their viability as judged by the trypan blue exclusion test. In vivo hyperthermia has no significant effect on spleen cell cytotoxicity (Table 3).

**DISCUSSION**

The major conclusions that can be drawn from the data are: (a) local heating of a primary tumor for two 120-min sessions, such that intratumor temperature is maintained at 42.3°C for 90 min at each session, inhibits the growth of both the primary and a preexisting metastasis distant from the site of therapy; (b) heating inadequate to reduce growth rate of a primary tumor does not result in enhanced growth of metastases; and (c) local heating adequate to inhibit tumor growth has no effect on the host's cell-mediated immune response in an in vitro assay.

We found that local hyperthermia applied by immersing the tumor-bearing limb in a water bath can cause reduced growth rate of primary, as well as decreased weight of a retroperitoneal metastasis when compared with sham-heated controls. This adds to the body of evidence showing that local hyperthermia can be therapeutic with respect to tumors distant from the site of therapy (3, 9, 15).

Dickson and Ellis (5) reported that local heating of a Yoshida tumor in rats, inadequate to induce regression, causes enhanced spread of metastases. In other communications from their laboratory (4, 6, 7), it was reported that heating of the Yoshida sarcoma in rats of the VX-2 carci-
Hyperthermia, Tumor Growth, and Immunity

Table 2

Inhibition of cytotoxicity by in vitro heating of spleen cells

Spleen cells from rats bearing a barely palpable Me-H tumor were heated in vitro by 1-hr immersion in a hot water bath at the indicated temperature. Immediately after heating, the cytotoxic capacity of the spleen cells toward Me-H tumor cells and allogeneic BP-11 tumor cells was tested.

<table>
<thead>
<tr>
<th>Experiment 1</th>
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<th>Experiment 2</th>
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<tbody>
<tr>
<td>Me-H target cells</td>
<td>BP-11 target cells</td>
<td>Me-H target cells</td>
<td>BP-11 target cells</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td><strong>cpm</strong></td>
<td><strong>% reduction</strong></td>
<td><strong>cpm</strong></td>
</tr>
<tr>
<td>21-23°</td>
<td>1,358 ± 189</td>
<td>66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5,351 ± 454</td>
</tr>
<tr>
<td>37.0</td>
<td>1,529 ± 304</td>
<td>62</td>
<td>5,036 ± 479</td>
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<td>39.5</td>
<td></td>
<td></td>
<td>3,950 ± 706</td>
</tr>
<tr>
<td>40.5</td>
<td>3,137 ± 428</td>
<td>22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6,582 ± 225</td>
</tr>
<tr>
<td>41.5</td>
<td>3,878 ± 216</td>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6,840 ± 1122</td>
</tr>
<tr>
<td>Media control</td>
<td>4,032 ± 779</td>
<td>6&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> In relation to media control.
<sup>b</sup> Mean <sup>3</sup>H cpm of 6 samples ± S.D.
<sup>c</sup> Difference between percentage of reduction of pairs identified with superscript letters are significant at p: c-g, <0.001; g-i, <0.05; f-j, <0.001; d-h, <0.02; and e-k, <0.05.

Table 3

Effects of in vivo hyperthermia on spleen cell cytotoxicity

Rats bearing Me-H tumor were subjected to 2 sessions, on consecutive days, of local hyperthermia to tumor-bearing limb. Either 1 or 2 days after the last hyperthermia session, spleens were removed for assay. Measurement was made of spleen cell cytotoxicity toward specific and nonspecific tumor target cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Me-H target cell</th>
<th>BP-11 target cell</th>
<th>Me-H target cell</th>
<th>BP-11 target cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Day posthyperthermia</td>
<td>48.3 ± 18.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.0 ± 5.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61.8 ± 14.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.8 ± 2.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 Day post-sham hyperthermia</td>
<td>70.7 ± 5.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.0 ± 7.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64.7 ± 6.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.0 ± 6.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 Days posthyperthermia</td>
<td>71.8 ± 3.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.0 ± 4.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.0 ± 11.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.5 ± 7.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 Days post-sham hyperthermia</td>
<td>74.3 ± 6.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.0 ± 7.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.3 ± 19.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.3 ± 16.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean percentage of cytotoxicity (relative to media control) ± S.D.
<sup>b</sup> Numbers in parentheses, number of spleen donors. Each spleen was assayed separately and 6 replicate assays were done with each spleen.

noma in rabbits sufficient to cause complete regression of the primary also results in eradication of preexisting metastases. In vitro studies from their laboratory also showed that low levels of heat increase the metabolism of tumor cells (4, 6). They therefore postulated that inadequate heating alters tumor cells in a way that leads to enhanced spread of tumor cells.

We, however, find that if heat is applied to a primary tumor for a single 2-hr session or if the leg contralateral to the tumor-bearing limb is heated for 1 or 2 such sessions, there is no effect on the growth rate of either the primary or metastases. Furthermore, Yerushalmi (18) reported that local hyperthermic treatment of the Lewis lung carcinoma-bearing limbs of mice leads to a delay in appearance of lung metastases, even though the growth rate of the primary tumor is unaffected.

A way to reconcile our and Yerushalmi's (18) data with that of Dickson and Ellis (5) may reside in a consideration of core body temperatures of hyperthermic-treated animals. Dickson and Ellis (5) immersed the tumor-bearing limbs of rats in a water bath to maintain an intratumor temperature of 42°, with the central body temperature rising to an average slightly over 41.5° for 60 min. Thus, the core body temperature applied by these workers was about a degree higher than that used in this communication, and this regimen led to enhanced metastasis. It seems possible that the level of heat used by Dickson and Ellis (5) was sufficient to impair vital systems of the host, including the immune system, since about 90% of their rats died during heating or within the next 24 hr. In other communications from their laboratory (4, 6, 7), it was reported that when hyperthermia was applied so that the level of heat was not life-threatening, regression of both the primary tumor and metastases could be effected. In these latter series of experiments, intratumor temperatures were maintained at 42° or higher for 1 or 2 hr but the core temperature of the host did not exceed 41°. Similarly, Yerushalmi (18) reported that the treatment of Lewis lung carcinoma in mice by whole-body hyperthermia leads to more rapid metastasis than in untreated controls, and suggested local heating may prove clinically more efficacious than whole-body heating. He also reported that local hyperthermia leads to a delay in metastasis. With local heating, body temperature was lower than with whole-body heating, although the 2
modes of therapy generated roughly equivalent primary intratumor temperatures. This suggests that the enhanced spread of metastases in response to hyperthermia probably does not result from inadequate heating of the tumor, but rather from overheating of the host.

It is not clear whether the inhibitory effect of hyperthermia on growth of metastases reported in this communication is a manifestation of an immune response triggered by hyperthermia. We found that heating tumor-bearing rats in vivo has no effect on spleen cell-mediated cytotoxicity toward specific tumor cells as tested in an *in vitro* assay. Heating was such that the temperature in the transverse colon adjacent to the spleen was raised to 40.7° for 1 hr. Spleen ther mia. We found that heating tumor-bearing rats in vivo to 40.5° for 1 hr, however, exhibit a diminished capacity for cell-mediated cytotoxicity. Harris (10) reported a similar effect of *in vitro* heating. If our *in vivo* heating regimen raises intrasplenic temperature to a level approximating 40.5°, this would indicate that an effect of heat on cell-mediated cytotoxicity either does not occur *in vivo* or can be repaired *in vivo*. In contrast to the suggestion by Harris (10), the data indicate that the maximal level of hyperthermia consistent with viability of the host has no adverse effect on this facet of the tumor immune response.

In our hands hyperthermia has no direct effect on the immune system sufficient to influence tumor growth. This is evidenced by the fact that heat applied to the limb contralateral to the tumor-bearing leg has no effect on growth rate of either the primary or secondary tumor. Hyperthermia may, however, have an indirect stimulatory effect on host immune response by causing increased antigenicity in heated tumor cells (12). Experiments are now in progress to determine whether the inhibition in growth of metastases observed when the primary tumor is heated is also observed in rats immunosuppressed by whole-body X-irradiation.

**REFERENCES**


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