Role of Cell-mediated Immunity in Tumor Eradication by Hyperthermia

Alan A. Alfieri, Eric W. Hahn, and Jae Ho Kim

ABSTRACT

A single hyperthermic treatment (44.5° for 20 min) which results in successful local control of a 7-day intradermally growing fibrosarcoma in intact mice fails to permanently control tumor growth in immunodeficient nu/nu (BALB/c) or immunosuppressed (whole-body radiation, 500 rads) mice. Studies were designed to determine whether humoral or cellular-mediated factors were responsible.

Nude mice were reconstituted with either heterozygotic (nu/+ ) sensitized or nonsensitized splenic T-cells. Similarly, semisyngeneic immunosuppressed BALB/c × C57BL/6 F₁ hybrids were reconstituted with homologous sensitized and nonsensitized T-cells or sera prior to hyperthermic treatment of a 7-day intradermal Meth-A implant. Successful local tumor control by hyperthermia was effected in those animals reconstituted with ≥1.8 x 10⁷ splenic T-cells but not in those with sera. The inhibition of macrophage activity (chronic silica, i.p.) could substitute for whole-body radiation immunosuppression.

These studies indicate that a thermally induced tumor cure would appear to be mediated by an activated macrophage-antigen-T-cell interaction which may be dependent on the initial expression of cell-mediated antitumor immunity that may be generated only in response to immunogenic tumors.

INTRODUCTION

It is generally agreed that tumor control in situ by localized hyperthermia (temperatures in excess of 40°) is dependent on time-dose relationships and the uniformity of heating (5, 6, 8, 21, 22). Additional factors suspected of influencing the quantitative variations observed are histopathology (21), tumor antigenicity (2, 6, 10, 18, 26), and the oxygen or pH status (5, 22) at the time of thermal treatment. Recent reports from this laboratory demonstrated that both site of growth (8, 10) and tumor age-dependent and/or host-related factors (2, 9, 10) appear to be of greater influence on tumors treated by heat alone. We have speculated that, when all optimal conditions were met, the loss of thermal responsiveness seen in experimental tumors might be explained by either: (a) their increasing invasiveness to underlying tissues; or (b) the inability of the host to generate an adequate level of specific concomitant immunity (2, 9, 10). While the reasons for the former are presently under investigation, the latter has not yet been fully evaluated as a contributing factor in the local eradication by heat alone of transplantable animal tumors.

Host-mediated interactions have been shown to influence tumor growth in situ. Similarly, hyperthermia (fever or 39–40° in vitro) and cell-mediated immunological responsiveness when assay- ing mitogen and/or antigen reactions (3, 17, 23, 26, 27) or increased bactericidal functions of phagocytic leukocytes (23). Thus, immunological participation in the curative response of tumors to localized hyperthermia either via direct stimulation of immune defense mechanisms or perhaps through the perturbations of the tumor cell surface (indirectly resulting in increased tumor-specific immunity of the host) should be considered as the possible mechanism for the tumoricidal action of heat. Although these possibilities have not been adequately confirmed, these may indeed be the delayed mechanisms postulated by several laboratories (4, 6, 7, 18, 26) as being partially responsible for the different control rates observed when murine tumors of varying antigenic intensities were subjected to comparable thermal treatments. Intrigued by these conflicting findings and the paucity of information on hyperthermia and host-related factors, we attempted to assess the role of a preexisting state of cell-mediated antitumor immunity as a primary requirement for local tumor control by heat alone.

MATERIALS AND METHODS

Animals. Syngeneic 8 to 10 week-old male BALB/c (H-2b) mice (Charles River Breeding Laboratories, Wilmington, Mass.) and semisyngeneic 8 to 10-week-old male (H-2d<sup>α</sup> × b BALB/c × C57BL/6 F₁ (hereafter called CB6F₁) mice (The Jackson Laboratory, Bar Harbor, Maine) were used in these studies. Male athymic nude (nu/nu) and heterozygotic (nu/+ ) mice of the genotype BALB/c (Grand Island Biological-Sprague-Dawley, Madison, Wis.) were used for these studies. The athymic animals were isolated in sterile plastic filter-covered cages and maintained on autoclaved food pellets and biweekly acidified (pH 2.5) or ampicillin-supplemented sterilized drinking water.

Tumor. The Meth-A tumor, a methylcholanthrene-induced fibrosarcoma which is highly antigenic for its syngeneic hosts (13, 20), was harvested from donor mice in the ascites form. The tumor cell suspension was diluted in minimal essential medium (Earle’s balanced salts) to a final concentration of 7.0 ± 0.5 (S.D.) x 10⁵ cells/0.1 ml and was agitated constantly by a magnetic spin bar as described by Alfieri and Hahn (1). Viability of the single-cell suspension was regularly >99% as determined by trypsin blue dye exclusion. All animals (except nude) were subjected to depilatory cream on the right hind limb 24 hr prior to inoculation. A tumor inoculum of 0.05 ml was injected i.d. into the thigh portion of the right hind leg with a 29-gauge needle on Day 0.

Tumors were palpated on alternate posttreatment days, and animals were observed for survival. Survival to Day 120 without evidence of disease was the end point chosen for evaluation. Autopsies were performed on animals whose early deaths were not attributed to the primary tumor, and histological preparations of the treated limb were examined microscopically for the...
presence of viable tumor tissue.

**Non-specific Immunosuppression.** Whole-body irradiation was given 48 hr (Day −2) prior to tumor inoculation with either a $^{137}$Cs Gammacell unit (AEC Ltd.) at a dose rate of 122 rads/min or, where specified, with a General Electric Maxitron X-ray machine (300 kVp, 20 ma, half-value layer of 1.88 at 2.0-mm copper filtration and 50-cm target-to-skin distance) at a dose rate of 126 rads/min. The total dose was 500 rads in both cases.

**Heat Procedure.** The water bath heating procedure has been described in detail elsewhere (1, 9). Briefly, LTH was achieved by immersing the tumor-bearing appendage for 20 min in a water bath heated to 44.6 ± 0.05° by a Haake Model E-52 temperature circulator (Saddlebrook, N. J.). The temperature was standardized to an NBS thermometer. Tumors grown i.d. were then processed according to the method of Julius et al. (12) for T- and B-cell enrichment. Briefly, 0.6 g of dry nylon wool (Leuko-Pak Leukocyte Filter, Fenwal Laboratories, Deerfield, Ill.) was rinsed with 37° phosphate-buffered saline (No. 310-4190, Grand Island Biological Corp., Grand Island, N. Y.) containing 5% heat-inactivated FCS. These cells were then processed according to the method of Julius et al. (12) for T- and B-cell enrichment. Briefly, 0.6 g of dry nylon wool (Leuko-Pak Leukocyte Filter, Fenwal Laboratories, Deerfield, Ill.) was rinsed with 37° phosphate-buffered saline with 5% FCS prior to cell packing. Spleen cells were applied to the sterile plastic syringe columns, washed into the wool, and incubated for 90 min at 37°. All nonadherent cells collected in the effluent were pooled, spun at 1200 rpm, and resuspended to appropriate concentrations. These cells were assayed for viability and surface marker [anti-Thy 1.2 sera (1:20) and lethally irradiated rabbit complement (1:20) for cytolyis as determined by trypan blue dye exclusion] prior to retroorbital sinus inoculation into immunosuppressed recipients.

**Sera.** For preparation of sera, mice were anesthetized with ether and bled aseptically by heart puncture. The blood was spun at 5000 rpm for 10 min, and the respective sera were pooled and stored at −20°. Syngeneic sera were prepared from mice maintaining 7- to 15-day Meth-A implants and were defined as control sera. Sera and sensitized lymphocytes were also prepared either from animals that were free of disease for more than 60 days after hyperthermic treatment of 7-day i.d. implants or from mice rendered immune to Meth-A by 2 separate i.p. inoculations given 14 days apart of 10⁶ homologous tumor cells that had been given 10 kilorads. Animals immunized by either method were found to have sera that were negative for antitumor antibodies by the method of indirect immunofluorescence using fluorescein isothiocyanate-conjugated antimouse IgG-IgM against 10⁶ Meth-A target cells. Despite the apparent absence of humoral antibody to Meth-A, these animals were capable of rejecting tumor cell challenge, thus demonstrating their immune status. These sera were pooled and defined as immune sera from immunized animals. Prior to inoculation into genetically matched recipients, all sera were thawed at room temperature and passed through a 0.45-μm Millipore filter.

**RESULTS**

The percentage of primary local tumor control when LTH (44.5° for 20 min) was administered to a 7-day-old i.d. grown Meth-A fibrosarcoma is summarized in Table 1. Tumors grown in normal BALB/c, syngeneous nu/nu (BALB genotype) and semisyngeneic CB6F1 mice were responsive to one hyperthermia treatment (>63% survival) as has been reported previously. In all animals, hyperthermia-induced hemorrhagic changes were observed in the tumors within hours after treatment, and this was followed by extensive necrosis within 24 hr. Within 14 days, the necrotic tumor sloughed, and the wound healed by Day 21; this resulted in long-term cure without compromise of normal tissue elements. Tumors in comparably treated animals rendered nonspecifically immunosuppressed by whole-body radiation 48 hr prior to implantation or in congenitally immunodepressed athymic mice proved to be refractory to long-term cure by single hyperthermic treatment (Table 1). These tumors also showed extensive early necrosis followed by regression. However, a ring of viable tumor tissue emerged around the necrosis at approximately 2 weeks posttreatment and grew progressively, subsequently killing the host.

To determine whether the presence of host factor(s) was required for tumor control by localized hyperthermia, normal mice and whole-body-irradiated (suppressed) animals were inoculated i.v. with syngeneic sera on the fifth day after i.d. tumor implant. Neither control sera nor sera from immunized animals augmented the immunosuppressive blockade of Day −2 irradiation (Table 2). Primary tumor control was found to be unaffected at either 0.25 or 0.5 ml of serum per mouse. Similar results were obtained for nonspecific immunosuppression by whole-body irradiation when animals were given a macrophage-specific inhibitory agent. Multiple i.p. inoculations of fumed silica, administered simultaneously at time of implantation (Day 0) and repeated 4 times at 48-hr intervals, abrogated the curative response of a single hyperthermic treatment (Table 2). A single i.p. inoculation at Day 0 (data not shown), however, was not effective in abrogating this response.

**Identification of Donor Cells Necessary to Overcome the Non-specific Immunosuppressive Blockade.** Since curative effects of hyperthermia were shown to be completely abrogated in T-cell-deficient animals or in those given sublethal whole-body irradiation prior to tumor inoculation, it was conceivable that a radiosensitive host factor may play a major role in local tumor control by heat alone. We therefore undertook to determine whether sensitized or nonsensitized donor T-cells could

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* P. J. Higgins and A. A. Alfieri, unpublished data, 1980.
* S. Green, personal communication.
Survival following localized tumor hyperthermia treatment of an i.d. Meth-A fibrosarcoma implant

Tumor was placed in a water bath at 44.6 ± 0.05°C (S.D.) for 20 min on Day 7 of growth. The implant was a 7-day tumor grown i.d. (inoculated on Day 0 as 3.5 ± 0.25 × 10⁵ cells/0.05 ml).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Immune status</th>
<th>No. of survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB6F,</td>
<td>Intact</td>
<td>12/19 (63)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Intact</td>
<td>7/11 (64)</td>
</tr>
<tr>
<td>nu/nu (BALB/c)</td>
<td>Intact</td>
<td>11/11 (100)</td>
</tr>
<tr>
<td>CB6F,</td>
<td>500 rads WB</td>
<td>0/21 (0)</td>
</tr>
<tr>
<td>BALB/c</td>
<td>500 rads WB</td>
<td>0/9 (0)</td>
</tr>
<tr>
<td>nu/nu (BALB/c)</td>
<td>Athymic</td>
<td>0/11 (0)</td>
</tr>
</tbody>
</table>

# Numbers in parentheses, percentages.

Table 1

Survival following localized tumor hyperthermia treatment of an i.d. Meth-A fibrosarcoma implant

Tumor was placed in a water bath at 44.6 ± 0.05°C for 20 min (Day —7). The implant was a 7-day tumor grown i.d. (inoculated on Day 0 as 3.5 ± 0.25 × 10⁵ cells/0.05 ml).

<table>
<thead>
<tr>
<th>Immune status</th>
<th>Treatment</th>
<th>No. of survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>Control</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>500 rads WB</td>
<td>Control</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Intact</td>
<td>Immune</td>
<td>5/6 (85)</td>
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<tr>
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<tr>
<td>Intact</td>
<td>Silica</td>
<td>0/7 (0)</td>
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<tr>
<td></td>
<td>Fused silica</td>
<td>1/9 (11)</td>
</tr>
</tbody>
</table>

# Sera inoculated on Day 5 (0.25 to 0.5 ml into retroorbital sinus); see "Materials and Methods."

Table 2

Survival following localized tumor hyperthermia treatment of an i.d. Meth-A fibrosarcoma implant

Tumor was placed in a water bath at 44.6 ± 0.05°C for 20 min. The implant was a 7-day tumor grown i.d. (inoculated on Day 0 as 3.5 ± 0.25 × 10⁵ cells/0.05 ml).

<table>
<thead>
<tr>
<th>Cell-mediated Immunity and Hyperthermia</th>
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</table>

DISCUSSION

In the present experiments, we have attempted to study whether cellulary mediated events are the fundamental mechanisms associated with tumor eradication by hyperthermia. The inability of sera from immunized animals to abrogate the nonspecific immunosuppressive blockade (500 rads, whole-body irradiation) suggested limited involvement of humoral elements, at least in the early phase of posthyperthermic tumor cell destruction. It appears from these studies that B-lymphocytes may not be necessary in the absence of a preexisting immune effector mechanism for initiating tumor regression. These experiments do not specifically indicate whether T-cell-enriched lymphocytes were the direct mediators of the cytotoxic effects or were effectors by other indirect mechanisms. For example,

be to overcome a radiation-induced or congenital blockade in recipient animals. Initial experiments indicated that reconstitution with less than 3 × 10⁶ nonsensitized T-cells by retroorbital inoculation immediately after tumor implantation (Day 0) was not effective in promoting primary tumor control posthyperthermic treatment (data not shown).

When animals previously suppressed by whole-body irradiation (Day —2) were reconstituted with 2.5 × 10⁷ (Day 0) or 5.2 × 10⁷ (Day —1) Meth-A-sensitized (CB6F,) T-cells prior to localized hyperthermic treatment, permanent tumor regression within 48 and 24 hr after irradiation-induced blockade, localized tumor control was also seen in 36% (5 of 14) and 89% (8 of 9) of the animals (Table 3). Thus, the ability to adoptively transfer tumor rejection or enhance primary tumor control after hyperthermia with sensitized lymphocytes could be demonstrated. However, when semisyngeneic CB6F, mice were reconstituted with 2.5 × 10⁷ (Day 0) or 5.2 × 10⁷ (Day —1) nonsensitized (CB6F,) T-cells within 48 and 24 hr after irradiation-induced blockade, localized tumor control was also seen in 36% (5 of 14) and 89% (8 of 9) of mice after tumor hyperthermia (Table 3). These results suggest that hyperthermia-induced tumor control was more dependent on absolute lymphocyte numbers than on the immune status of the lymphocytes which were inoculated.

It can be shown from these preceding experiments on "normal" mice that reconstitution with T-cell-enriched populations could effectively reverse the radiation-induced immunosuppressive blockade, resulting in localized hyperthermic tumor control. It was important, however, to demonstrate this effect in both normal and congenitally athymic animals in order to eliminate the possibility that latent thermal cures were a result of hematopoietic regeneration or partial restoration of immunocompetence at the time after treatment when heated tumors may have had minimal numbers of clonogenic cells. It was the purpose of the following experiments, therefore, to determine whether tumors in congenitally athymic animals previously found to be refractory to thermal cure could be controlled by restoring them to immunocompetency with a T-cell-enriched splenic cell population. This would represent additional evidence that generation of immunity is a prerequisite for thermally induced tumor cure.

Results from these experiments are shown in Table 3. When homozygous nude mice (nu/nu) were reconstituted with 2.5 × 10⁷ (Day —3) or 1.8 × 10⁷ (Day —1) heterozygous enriched T-lymphocytes (from disease-free animals) prior to i.d. Meth-A tumor implant, primary tumor control by hyperthermia given on Day 7 was demonstrated by 75 and 62% survival, respectively. This ability to adoptively transfer tumor-specific immunity in nude mice is consistent with the results obtained with the semisyngeneic CB6F, hybrids. Independent of this ability to adoptively transfer immunity, when nude homozygotic mice were reconstituted with nonsensitized heterozygotic (BALB/nu) T-lymphocytes on Day —3 (2.5 × 10⁷ cells) or Day —1 (1.9 × 10⁷ cells) prior to LTH (Day 7), primary tumor control of 25 and 50% could also be demonstrated.
the possibility exists that collaboration with macrophages or B-cells via contaminant populations from glass wool columns (10 to 15%) or radioresistant fractions could effect antibody production. One line of evidence supporting macrophage involvement in the posttreatment of thermally damaged cells was the significant reduction in thermal cures observed when multiple i.p. silica inclusions (known to transiently inhibit macrophage function9) were administered to Meth-A tumor-bearing recipients. Although this evidence is not conclusive by itself, it suggests that an activated macrophage-antigen-T-cell processing is necessary for complete tumor cell destruction by heat alone in this tumor system.

To date, there exist only a few reports providing information that curative hyperthermia will stimulate both cellular and humoral immune responsiveness against a host tumor. Abacopal effects have been reported in both experimental systems (6, 7, 28) and in patients whose hyperthermia treatments of one tumor site resulted in partial regression of untreated lesions (4). When rabbits maintaining VX2 carcinomas were given localized heat (6, 26), both antibody titers and cell-mediated cytotoxicity were found to be either elevated or not adversely affected as in the spleen cell-mediated, tumor-immune response of rats (24). However, many of these effects are nonspecific for the tumor, or they have been seen with other therapy regimens and have not been shown to be uniquely augmented by different treatment modalities, provided tumor removal (or destruction) is nearly complete. In contrast, it has been shown that direct heating of isolated murine spleen cells to cytotoxic temperature of 43° for 45 min can rapidly inactivate cytotoxic thymus-derived lymphocyte function on a mixed-lymphocyte culture assay (11). Subsequent work, however, has shown that cytotoxic thymus-derived lymphocyte reaction to this temperature is acute and reversible with reincubation at 37° between split doses (17). Recovery was found to be temperature-dependent in addition to the observed increases in lytic activity, increasing approximately 40-fold when mixed-lymphocyte cultures were incubated for >1.5 hr.

It is possible, nevertheless, that perturbations of the tumor cell surface resulting from hyperthermic treatment may indeed increase the tumor-specific immunity of the host. Although these possibilities have not been fully confirmed, earlier reports in the literature have alluded to an earlier onset of the immune response, as revealed by lymphoblast appearance in mesenteric lymph nodes when Ehrlich adenocarcinoma cells were heat inactivated rather than heavily irradiated (23). This may account for the increased immunogenicity seen when Ehrlich ascites cells were exposed to mild hyperthermia by Mondovi et al. (19). In their studies, heat-killed (42.5° for 3 hr) cells protected mice from tumor inoculation to a greater level than did X-ray-inactivated tumor cells. However, subsequent studies by Suit et al. (28) with the FSa fibrosarcoma could not corroborate the immunological protection afforded by heat-inactivated tumor cells when compared to that achieved by irradiation.

The factors that determine whether hyperthermia may enhance the immune response are not certain, but they are likely to involve such variables as the mode of antigen presentation (cell bound or free), immunogenic potential of the antigen, and the possible alteration that hyperthermic therapy may contribute to the initial site(s) of interaction or at the cellular level of the responding host. While it may be hazardous to extrapolate our data to hyperthermic clinical trials (14–18), it is of interest that human tumors metastatic to skin in which regressions have been transient cannot be permanently controlled by heat alone. Thus, human tumors that are relatively nonimmunogenic may not be capable of presenting adequate antigenic stimulus for concomitant T-cell-mediated antitumor immunity necessary for hyperthermia-induced tumor cure. Indeed, a future direction for hyperthermic investigations that may be the most promising, as shown by Szimigiel and Janiak (29), is the application of both specific and nonspecific immunostimulation and hyperthermia therapy.

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