Enhancement of Thermal Response of Normal and Malignant Tissues by Corynebacterium parvum

M. Urano, T. Yamashita, H. D. Suit, and L. E. Gerweck

Edwin L. Steele Laboratory for Radiation Biology, Department of Radiation Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114

ABSTRACT

Further studies were carried out on the combined effects of Corynebacterium parvum and hyperthermia on animal tissues and cultured Chinese hamster ovary cells. Experimental animals were C3Hf/Sed mice derived from our defined flora mouse colony. Tumors were eighth-generation isotransplants of a spontaneous fibrosarcoma, FSa-I. Hyperthermia was given by immersing the mouse foot or culture flasks in the constant temperature water bath. Present experiments include thermal enhancement of C. parvum at different temperatures, effect of the agent on the kinetics of thermal resistance, and the mechanism of the thermal enhancement.

The thermal enhancement by C. parvum was independent of temperature in a range between 42.5 and 46.5°C, and it increased with decreasing temperature. The analysis of the Arrhenius plot suggested a comparable activation energy for combined treatment and for heat alone between 42.5 and 46.5°C. The thermal resistance developed very rapidly in both normal and tumor tissues. Systemic administration of C. parvum failed to modify the kinetics of thermal resistance.

Several experiments were attempted in order to disclose the mechanism. A single injection of C. parvum-induced macrophages failed to enhance thermal response of the mouse foot, while 3 daily injections of the macrophages enhanced the response, indicating that the enhancement by C. parvum is at least partly attributed to the C. parvum-induced macrophages. Whole-body irradiation of 6 Gy and/or administration of anti-mouse T-cell serum and histamine failed to inhibit the C. parvum enhancement of thermal response. No thermal enhancement was observed for Chinese hamster ovary cells treated at 43.0°C in vitro with C. parvum or thiomersalate, a preservative supplemented in C. parvum, although cytotoxic effect was shown at a high concentration of thiomersalate.

INTRODUCTION

Systemic administration of Corynebacterium parvum, killed anaerobic bacteria, enhanced the response of murine tissues to the elevated temperature (29, 30). This enhancement was greater for tumor eradication than for normal tissue response, resulting in a favorable differential effect between normal and malignant tissues. The therapeutic gain, which is a ratio of the enhancement ratio for tumor to that for normal tissue, was 1.1 for nonimmunogenic mouse fibrosarcoma and 2.3 for moderately immunogenic chemically induced mouse fibrosarcoma.

An essential difference lies between the action of C. parvum and that of hyperthermia. A moderate level of hyperthermia gives the lethal damage directly to the cell (3, 21, 24), and the treatment is probably given to the localized tumor. This effect is not specific on the tumor cells, but a rationale for use of hyperthermia in cancer treatment is that the hypoxic tumor cells, which are protected from radiation damage, are at least as sensitive as are well-oxygenated cells to the increased temperature (9, 18). The C. parvum has been reported to act specifically on tumor cells by potentiating the host immune reaction (1, 10). It could be effective not only to the localized area but to the disseminated lesions. Presumably, the systemic administration is a choice for C. parvum treatment (2).

The difference in the biological effectiveness of these agents offers an attractive feature for potential use of combined C. parvum and heat treatments. Therefore, further studies have been achieved on the effect of a systemic administration of C. parvum on the thermal response of normal and tumor tissues in experimental animals. We have attempted to disclose the mechanism of the C. parvum-induced thermal enhancement.

MATERIALS AND METHODS

In Vivo Experiments. Animals were 10- to 12-week-old C3Hf/Sed mice derived from our defined flora colony (22). They were kept in our animal facility, where defined flora conditions have been maintained. Sterilized Wayne Lab Blox and acidified and vitamin K-fortified water were provided ad libitum. Tumors were eighth-generation isotransplants of a fibrosarcoma which arose spontaneously in a C3H/Sed female mouse. This tumor was designated FSa-II.

The single-cell suspension was prepared by the use of a trypsinization technique that has been fully described elsewhere (28). This suspension, containing an adequate number of viable tumor cells (in 5 μl), was transplanted into the right foot. Hyperthermia was achieved by immersing the mouse foot into the water bath maintained at desired temperature ± 0.1°C (range). The tumor temperature was equilibrated in 90 sec and was not greater than 0.1°C below the water bath temperature.

C. parvum was kindly supplied by Burroughs Wellcome and Co. (Research Triangle Park, NC) as formalin-killed organisms suspended in 0.01% thiomersalate solution or in saline. A dose of 350 μg/mouse was injected i.v. 3 days before hyperthermia unless otherwise stated, since this dose can produce the maximum effect without unfavorable toxicity (23). Thiomersalate, the preservative supplemented in C. parvum suspension, was purchased from Eastman Kodak Co. (Rochester, NY). Anti-mouse T-cell serum was obtained from Cedariane Laboratories Ltd. (Hicksville, NY) and reconstituted with distilled water immediately before use.

Macrophages were collected by giving an i.p. administration of 1 mg of C. parvum or 2 ml of thiglycolate (Difco Laboratories, Inc., Detroit, MI), which was followed by the peritoneal washing 7 days later. These cells are termed C. parvum-induced or normal macrophages, respectively.

1 This work is supported by Grants CA26350 and CA13311 awarded by the NIH, Department of Health, Education, and Welfare.
2 To whom requests for reprints should be addressed.
3 Present address: Department of Radiology, Jikei University of Medicine, Nishishinbashishi, Minato-ku, Tokyo, Japan.
4 Andre Soriano Director of Cancer Management, Massachusetts General Hospital.
The blood pH was measured by a BM53 Mk2 blood microsystem and a PHM71 Mk2 acid-base analyzer (Radiometer, Copenhagen, Denmark). Blood was taken from the orbital sinus of each mouse.

Local irradiation to the mouse foot was achieved by a $^{137}$Cs unit which is specifically designed for partial body irradiation with a fixed field with a diameter of 3 cm (13). Two sources were mounted in opposition, and the dose rate was $-8.5$ Gy/min. The WB$^F$ was made by another cesium unit which possesses a larger field, with a dose rate of approximately 1.0 Gy/min.

Tumor response was studied by analyzing the median TG time (25, 26). Animals were randomly assigned to groups after transplantation, and they received a treatment when tumors reached an average diameter of 6 mm. Three diameters of each tumor, a, b, and c, were measured daily before treatment and at least 3 times a week after treatment. The tumor volume was calculated as an ellipsoid by a formula of $\pi abc/6$ and were plotted on a semilogarithmic graph as a function of time after treatment. The TG time was determined on the growth curve of each tumor, and the median TG time, i.e., the time for one-half of the treated tumors to reach 1000 cu mm, was calculated by the logit analysis method. Seven to 10 animals were used/dose group.

Normal tissue response was studied by scoring the peak foot reaction or by the RDso assay. Before any treatment, the animals were randomized into groups. The early reaction of each mouse foot was scored between Days 6 and 35 after treatment, every day for the first 10 days with decreasing frequency for the subsequent 20 days because of relative rapid decay of early skin reaction. After a large dose, a permanent or irreversible damage followed severe skin reaction.

In vitro experiments. CHO cells were routinely cultured in plastic flasks with McCoy’s Medium 5A (containing 26 mM NaHCO3 plus 10% calf serum and 5% fetal calf serum. The cells were maintained at 37°C in humidified air containing 5% CO2. Under these conditions, the population-doubling time of exponential-phase cells was $\sim 14$ hr (8). The cells were routinely subcultured every 2 or 3 days. This cell line was chosen for in vitro study because of the lack of cell lines established from FSa-II tumor cells.

Cells were heated by total submersion of the flasks in an insulated water bath containing a heater circulator which maintained the temperature at 43.0 ± 0.1°C. Temperature equilibration in the flasks of the cell surface occurred in $\sim 2.5$ min. Following heat treatment, the cells were incubated for 7 to 11 days. After incubation, the colonies were rinsed with saline, fixed with 100% methyl alcohol, and stained with crystal violet. The fraction of cells giving rise to colonies containing >50 cells was normalized to the fraction of control cells giving rise to colonies. Four flasks were used to determine each survival point. The surviving fraction was determined after correcting for cellular multiplicity determined at the time of heat treatment (8). The cell survival curve after hyperthermia was expressed by $D_0$ and $n$.

RESULTS

Enhancement by C. parvum at Various Temperatures. Effect of C. parvum on the enhancement of thermal response of mouse foot was studied at various temperatures. Animals treated with or without C. parvum received local hyperthermia in their feet at 41.5-46.5°C 3 days later, and the foot reaction was scored. The RDso values for animals which received combined treatments were significantly lower than those for animals treated with heat alone (Chart 1), indicating that C. parvum enhanced the foot reaction to heat. Exponential relationships were found between the RDso and the temperature for both treatments. A breaking point was seen at $\sim 42.5°C$ on the regression line for hyperthermia alone, although no breaking point emerged after combined treatments. The enhancement ratio, i.e., the ratio of RDso (heat alone) to RDso (combined treatments), was $\sim 1.5$ between the temperature range of 42.5 and 46.5°C, and it increased with decreasing temperature.

In the same figure, the reaction rate, which is assumed to be proportional to the reciprocal of RDso, and the reciprocal of absolute temperature $T$ are indicated to obtain the Arrhenius plot. This will be discussed later in the Discussion section.

Effect of C. parvum on the Split Administration of Hyperthermia. It has been demonstrated that mammalian cells treated at elevated temperature obtain thermal resistance (thermotolerance) which reaches a maximum within 24 to 48 hr and subsides gradually (7, 12). This phenomenon has been observed in animal tumors and in normal tissues (15, 20).

The effect of a systemic administration of C. parvum on this process was examined in normal and malignant tissues. Animals with or without tumors received 2 equal heat doses with various $T$s. For the combined treatment, C. parvum was given i.v. 3 days before the first heat dose, and the treatment time was two-thirds of that for hyperthermia alone, which was expected to result in an isoeffect. In Chart 2, the average peak foot reaction and the TG time are plotted as a function of $T$. A rapid decrease...
in both the average peak foot reaction and TG time was observed in the first 48 hr which was followed by relatively stationary scores of the foot reactions with a subsequent increase of TG time. No appreciable difference in these relations was seen between animals treated with heat alone and those treated with combined C. parvum plus heat, indicating no significant effect of C. parvum on the kinetics of thermal resistance.

Mechanism Involved in the C. parvum Enhancement of Thermal Response. Some previous experiments were attempted, without success, in order to understand the mechanism involved in the C. parvum enhancement of thermal response. Further attempts have been made.

Effect of the C. parvum-induced macrophages on the thermal response of the animal foot was examined, since the macrophages were shown to play a significant role in the antitumor effect of C. parvum (1, 10, 33). A given number of C. parvum-induced or normal macrophages (5 x 10⁸) were injected i.v. or directly into the foot pad at various times before or immediately after hyperthermia. The average peak foot reactions are summarized in Table 1, which indicates an increase in the thermal response only when the macrophages were given i.v. 24 hr before local hyperthermia. However, repeated experiments failed to confirm this finding, as shown in the second and third rows. A question that arises is whether the timing of administration and the number of macrophages may influence the enhancement effect. Therefore, 3 daily injections of 4 x 10⁹ macrophages each were made, with the last injection given 2 hr before heating. The results from 2 experiments were summarized in Table 1, row 4, since both results were identical. It is clear that administration of massive C. parvum-induced macrophages enhanced thermal response of the murine foot.

The reaction of antigens with some tissue cells results in a release of several substances, including intracellular histamines, into the blood. The histamine is claimed to enhance the thermal response (4). If the C. parvum releases the histamine, it may enhance the thermal response, and an antihistamine might protect the C. parvum enhancement. The effect of histamine and antihistamine on the foot reaction was examined with or without C. parvum. Animals receiving histamine or antihistamine were treated with hyperthermia at 43.5° for 120 min. The score of average peak foot reaction demonstrates no significant enhancement by histamine and no inhibition of the C. parvum enhancement by antihistamine (Table 2). These results suggest no direct involvement of histamine-related reactions in the C. parvum enhancement.

The following series of experiments examined the effect of C. parvum in animals receiving WBI, which suppresses the hosts' immune response. WBI of 6 Gy was followed by a systemic administration of C. parvum 24 hr later. Subsequently, local hyperthermia was given 3 days after C. parvum injection. The WBI did not inhibit the C. parvum enhancement of thermal response (Table 3). The RD₅₀ in animals pretreated with the WBI...
and C. parvum was 99 min, which was not significantly different from that in animals pretreated with C. parvum alone, which was 91 min, while both values were significantly shorter than the control value of 133 min. This evidence was also confirmed in a repeated experiment, as shown in Table 3, Column 9. One group of animals in the repeated experiment received C. parvum only 20 to 40 min before local hyperthermia. They also exhibited a remarkably lower RDso than that of control animals, with no significant difference from that of animals receiving C. parvum 3 days before hyperthermia. The failure of WBI to suppress the C. parvum enhancement indicates no appreciable relation of immunological mechanism with the C. parvum enhancement. Table 3 also demonstrates that the WBI or foot irradiation which was given alone 4 days before local hyperthermia did not modify the RDso.

The effect of anti-mouse T-cell serum on the mouse foot reaction was examined with or without administration of C. parvum. A single i.p. dose of 0.1 ml of the serum, which sterilized ~35% of spleen lymphocytes, was followed by local hyperthermia with a Tt of 4 days. C. parvum was given 1 day after administration of the serum. The anti-mouse T-cell serum as observed after WBI did not modify thermal response of the foot nor inhibit the C. parvum enhancement of the thermal response (Table 4).

Effect of Thiomersalate In Vivo. The thiomersalate, a preservative supplemented in C. parvum preparation, includes mercuric ions which may react with some essential cellular enzyme and result in a critical damage (11). Animals received C. parvum with or without thiomersalate or thiomersalate alone, and their feet were treated at 43.5° for 120 min 3 days or immediately thereafter. Another group of animals received hyperthermia immediately prior to administration of such drugs. Increased response was observed only in animals pretreated with C. parvum, regardless of supplemental thiomersalate (Table 5). The thiomersalate itself showed no additional effect on the thermal response of the mouse foot.

Blood pH. The blood pH was measured following C. parvum injection. Animals receiving no C. parvum injection showed a pH value of 7.36 ± 0.04 (S.D.). The C. parvum given 30 min to 3 days before hyperthermia did not alter the pH value (Table 6).

Toxicity of C. parvum and/or Thiomersalate In Vitro. The cytotoxicity of C. parvum and thiomersalate was tested at 37° and 43°. Drugs were added to medium immediately after cell plating and were left until the termination of the experiment. Treatment at 43° was given for 1 hr, approximately 4 hr after cells were plated. Cell survival is presented in Charts 3 and 4 as a function of drug concentration. At both temperatures, C. parvum alone exhibited virtually no cytotoxic effect over the drug range tested, although 1 hr at 43° decreased the surviving fraction to 0.12. The slight decrease of the survival at a high drug concentration was not statistically significant (Chart 3). However, the thiomersalate exhibited a cytotoxic effect, particu-
Enhancement of Thermal Response by C. parvum

Chart 3. Cytotoxic effect of C. parvum on CHO cells at 37° and 43°. The agent was added to the cells immediately after plating and was left for 7 days, until the termination of the experiment. Surviving fraction is plotted as a function of C. parvum concentration in medium. D, •, treatment with C. parvum at 37° and 43°, respectively. Bars, S.D.

Chart 4. Cytotoxic effect of thiomersalate and C. parvum supplemented with thiomersalate on CHO cells at 37° and 43°. Drugs were added 4 hr before hyperthermia and were left until the termination of the experiment. O, •, treatment with C. parvum containing thiomersalate at 37° and 43°, respectively. D, treatments with thiomersalate alone at 37°. Note that C. parvum preparation supplemented with thiomersalate contains C. parvum and thiomersalate in a ratio of 70:1. Top and bottom abscissae are scaled in this ratio. Bars, S.D.

Chart 5. Effect of C. parvum containing 0.01% thiomersalate on the hyperthermic dose-cell survival curve. Drugs were added 4 hr before hyperthermia and were left until the termination of the experiment. Four different concentrations of drugs were tested. The concentrations of C. parvum and thiomersalate (μg/ml) are: O, 0; □, 0.75; ■, 0.13; △, 17.5; 0.25; and ●, 40.0, 0.57, respectively.

DISCUSSION

We have demonstrated that a systemic administration of C. parvum enhanced tissue response to hyperthermia. Proposed

particularly at doses greater than 0.30 μg/ml. The dose-response curve showed a large initial shoulder, followed by a steep decrease in survival. Treatment with C. parvum containing thiomersalate showed a cytotoxic effect similar to that seen with thiomersalate-alone treatment (Chart 4). The hyperthermia at 43° for 1 hr decreased survival to 0.2 in this experiment, while the cytotoxic effect of thiomersalate was independent of the temperature.

Combined Effect of C. parvum Containing Thiomersalate and Hyperthermia In Vitro. The effect of selected heat treatment on CHO cells in the presence or in the absence of C. parvum containing thiomersalate was examined. C. parvum containing thiomersalate was added to the medium 4 hr prior to hyperthermia and then left until the termination of the experiment. Chart 5 presents heat cell survival curves obtained in various concentrations of the agents. Hyperthermia treatment alone yielded a D0 (95% confidence limit) of 21 (17 to 28) min and an n of 4.3. The slopes of survival curves for combined treatments were independent of the drug concentrations; i.e., the D0 was found in a range between 20.5 and 23.8 min, although the drug treatment alone, as indicated by survival at 0 min of hyperthermia, produced an orderly dose-response relationship. These results indicated that C. parvum supplemented with thiomersalate did not enhance thermal response of CHO cells and that the combined treatments exhibited only additive effects.
M. Urano et al.

mechanisms include: (a) mechanisms involved in the expression of host-immune response; (b) inflammatory response against bacteria infection, such as fever; and (c) mechanisms which may directly or indirectly enhance the tissue response to heat. Our approach to the possible mechanisms was due to the deductive method rather than the direct proof.

In a previous paper, it was shown that C. parvum did not increase the body temperature or produce tissue hypoxia (25). Although it has been shown that the environmental pH modifies thermal sensitivity of mammalian cells (6, 8, 27, 31), no significant decrease was observed in the blood pH. Present studies demonstrated that administration of a large number of C. parvum-induced macrophages, which presumably play a significant role in the antitumor effect of this agent (1, 10, 33), enhanced thermal response of the murine foot (Table 1), indicating that the C. parvum enhancement is at least partly attributed to the C. parvum-induced macrophages. This finding has appeared to contradict our observation that the C. parvum enhanced the thermal response in the whole-body-irradiated animals, since the WBI inhibits some types of immune reactions (16). However, it is notable that the antitumor effect of C. parvum was not abolished by a WBI of 6 Gy (16). A present result that the C. parvum enhancement was not inhibited in animals receiving anti-mouse T-cell serum indicates no involvement of T-cells in the C. parvum enhancement.

We have examined the effect of histamine, a substance released following the reaction with antigens, on the thermal enhancement. Results indicated no causative role of this agent. A possible role of other types of immunological reactions, such as antigen-antibody complex-related reactions, has remained unanswered, although immediate development of the thermal enhancement strongly indicates that the involvement of this kind of reaction is unlikely.

Another possible mechanism might be the direct effect of C. parvum; i.e., C. parvum might enhance the thermal response by itself. However, our in vitro studies failed to demonstrate the direct effect of C. parvum (Charts 3 and 5). Namely, neither C. parvum nor thiomersalate, a supplemental preservative in C. parvum preparation, enhanced thermal response of cultured CHO cells. The in vivo studies shown in Table 5 were comparable to these in vitro studies.

The C. parvum preparation is supplemented with 0.01% thiomersalate as a preservative. This mercury-containing compound is capable of interacting with sulfhydryl-containing enzymes, with resultant perturbation in cellular metabolism (11). Although our in vitro results demonstrated a possible cytotoxicity of the agent at a concentration of greater than 0.2 μg/ml of medium, our animals tolerated an i.v. 40-μg/mouse dose of thiomersalate alone. Assuming a uniform distribution throughout the mouse body and a mouse body weight of 25 to 30 g, this dose might correspond to approximately 1.5 μg/ml of medium. These results indicated that the thiomersalate is not responsible for possible side effects of C. parvum (8, 9). More importantly, the present study denied a causative role of this agent in the C. parvum enhancement of thermal response.

The relationship between inactivation rate and temperature may be illustrated by an Arrhenius plot in which the logarithm of the inactivation rate is plotted as a function of reciprocal of the absolute temperature (32). In the present study, the inactivation rate may be proportional to the reciprocal of RD50. The activation energy (cal/mol) can be calculated from the slope of regression lines for the Arrhenius plot (14). Chart 1 demonstrates exponential relations in the temperature range between 42.5 and 46.5°C. Notably, the 2 curves shown are essentially parallel, indicating that, unlike combined radiation and heat treatment, with which activation energy was greater compared to heat alone (14), the preadministration of C. parvum did not alter the activation energy. In fact, it was found to be 135 and 148 kcal/mol for heat alone and C. parvum followed by heat, respectively. The activation energies for various tissues and cultured cell lines have been found in a range between 124 and 171 kcal/mol (5), which are comparable with present results.

The activation energy for heat alone was greater at temperatures below 42.5°C than at temperatures above 42.5°C. Although this breaking point has been observed at 43.0°C for cultured mammalian cells, that for most animal tissues has been found below 43.0°C (4, 14, 17, 19). This is further discussed in a separate paper (27). Interestingly, the Arrhenius plot for combined C. parvum and hyperthermia showed no breaking point in the temperatures between 41.5 and 46.5°C, indicating that, below 42.5°C, the C. parvum enhancement increases with decreasing temperature. The question of why the enhancement was greater at the temperatures below 42.5°C than at those above 42.5°C remains unanswered. Caution must be exercised in using C. parvum together with hyperthermia at temperatures below 42.5°C.

REFERENCES

18. Overgaard, J., and Bichel, P. The influence of hypoxia and acidity on the
Enhancement of Thermal Response of Normal and Malignant Tissues by *Corynebacterium parvum*


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/44/6/2341

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.