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Effect of Thermochemotherapy (Combined Cyclophosphamide and Hyperthermia) Given at Various Temperatures with or without Glucose Administration on a Murine Fibrosarcoma[1]

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ABSTRACT

The effect of combined cyclophosphamide (CY) and heat treatments on a murine tumor was studied at various temperatures. FSa-II tumors, the early generation isotransplants of a spontaneous fibrosarcoma in a C3Hf/Sed mouse, were used. A single cell suspension was transplanted into the animal foot. Hyperthermia was given by immersing animal feet into a water bath maintained at a desired temperature ±0.1°C. An average diameter of the tumor at the time of treatment was 4 mm. The tumor growth time, the time required for one-half of the treated tumors to reach 1000 mm^3, was the end point.

Hyperthermia enhanced the effect of CY at test temperatures ranging from 40.5°–44.5°C. The enhancement was independent of the temperature when CY was administered 30 min before the beginning of hyperthermia. However, the enhancement was most substantial at temperatures of 40.5°–42.5°C when CY was administered immediately before hyperthermia. The most effective timing of the CY administration was immediately before hyperthermia. The glucose administered 60 min before hyperthermia enhanced the effect of combined CY and hyperthermia when CY was given 30 min before heating. This enhancement was lost when CY was given immediately before hyperthermia. The CY dose response curves at elevated temperatures were downward concave, which may indicate the presence of a CY- and heat-resistant cell population in the tumor. Implications of these observations in clinical hyperthermia were discussed.

INTRODUCTION

It has been demonstrated in cultured mammalian cells and in animal tumors that hyperthermia can enhance the cytotoxic effect of some chemotherapeutic agents (1–5). We have shown enhanced cytotoxicity of CY[5] on our spontaneous fibrosarcoma (6). Our study indicates that thermochemotherapy is attractive since a substantial enhancement can be obtained at a low temperature (41.5°C) which induces no or very limited thermal damage in the tumor in the absence of chemotherapeutic agents. The same study also demonstrated that glucose administered 1 h before hyperthermia further enhanced the cytotoxic effect of CY. The enhancement of the drug cytotoxicity at low temperatures is obviously advantageous for human cancer treatment. A high temperature which induces thermal damage in a tumor frequently causes undesirable side effects (7, 8). The presence of a cold spot in a tumor which may be critical for the success of hyperthermia (9) could be eradicated by thermochemotherapy which is effective at low temperatures. Accordingly we have further studied the effect of combined CY and heat treatments on the tumor at various temperatures to ensure that the low temperatures (below 43.0°C) are appropriate for this type of treatment modality.

MATERIALS AND METHODS

Animals were 10- to 12-week-old C3Hf/Sed mice derived from our defined flora mouse colony. They were kept in our small animal facility where defined flora conditions have been maintained (10). Sterilized Wayne Lab-Blox and acidified, vitamin K-fortified water were provided *ad libitum*. Tumors were fourth to sixth generation isotransplants of a fibrosarcoma which arose spontaneously in a C3Hf/Sed mouse. Single cell suspensions were prepared from intact tumor tissues by trypsinization, and 5 μl of the suspension containing $\simeq 2 \times 10^5$ viable tumor cells were transplanted into the mouse foot. Further details are given elsewhere (11).

Hyperthermia was given by immersing animal feet into a water bath where a desired temperature ±0.1°C was maintained by a constant temperature circulator (Model B-1; Lauda, West Germany). Temperature in the tumor center was no less than 0.1°C below the water bath temperature (details are given in Ref. 12).

Tumor response was investigated by the TG time assay or the determination of the time required for one-half of the treated tumors to reach 1000 mm^3 from the initial treatment day. Namely, three diameters of each tumor were measured 3 times a week, and the tumor volume was determined by a formula, $\pi abc/6$. A growth curve was constructed to determine the TG time of each tumor, and the 50% TG time was calculated utilizing the logit analysis.

Test agent was CY (Mead Johnson & Co., Evansville, IN) which was dissolved in 0.9% NaCl solution (saline) immediately before use and injected i.p. Glucose (50% dextrose) was purchased from Elkins-Sinn, Inc., Cherry Hill, NJ, and was also given i.p.

RESULTS

The effect of thermochemotherapy with or without glucose administration was examined at various temperatures. Treatments were given when tumors reached an average diameter of 4 mm (tumor volume, 35 mm^3). A CY dose of 200 mg/kg was administered 30 min before the beginning of hyperthermia. A group of animals received a glucose dose of 5 mg/g 60 min before hyperthermia. TG time was determined as a function of treatment time at elevated temperatures (Chart 1). Although hyperthermia alone for 60 min did not prolong the TG time at

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[5] The abbreviations used are: CY, cyclophosphamide; TG time, tumor growth time (the time required for half the treated tumors to reach 1000 mm^3 from the initial treatment day).

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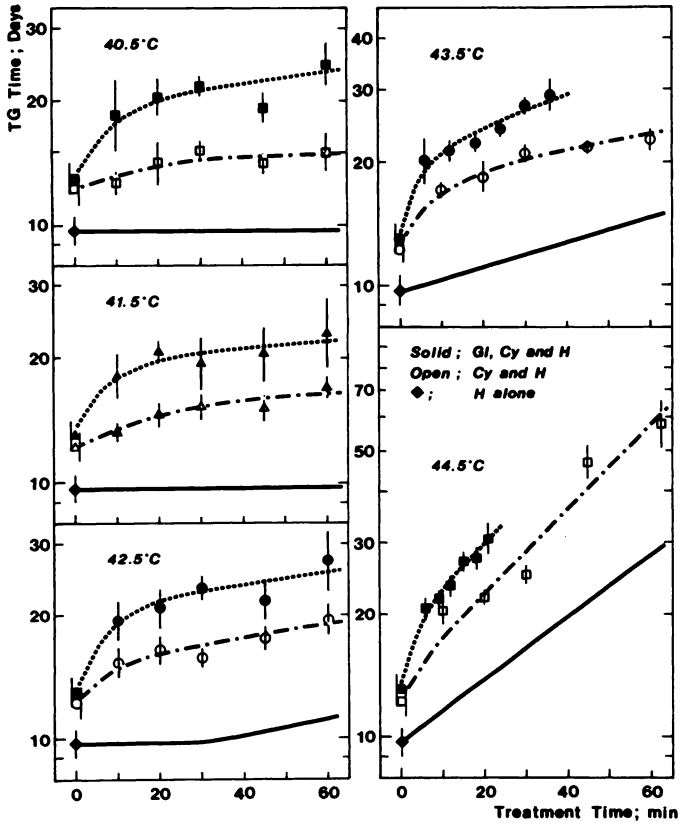

Chart 1. The effect of thermochemotherapy (combined CY and heat) with or without a glucose (*GI*) administration on the mouse FSa-II tumor at various temperatures. *Solid* and *open symbols* indicate combined CY and heat (*H*) given with and without glucose, respectively. A CY dose of 200 mg/kg was injected i.p. 30 min before hyperthermia. Glucose of 5 mg/g was given i.p. 60 min before heat treatment. Dose response curves for hyperthermia alone were taken from Ref. 22 and normalized. *Bars*, 95% confidence limits.

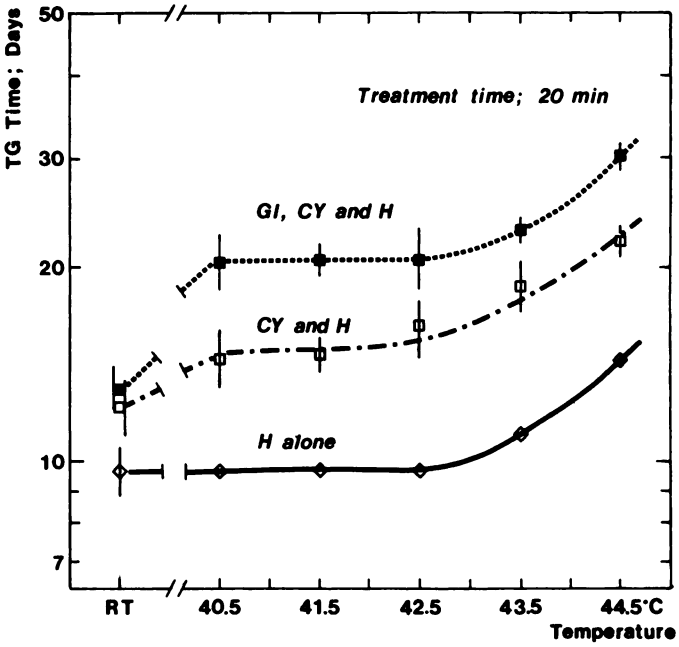

Chart 2. Comparison of the effect of thermochemotherapy with or without glucose (*GI*) at various temperatures. All data are taken from Chart 1. The ratio of the TG time [CY and heat (*H*)] to the TG time (heat alone) and the ratio of the TG time (glucose, CY, and heat) to the TG time (heat alone) are constant at the temperature of 40.5°–44.5°C. *RT* indicates room temperature at which the foot tumor temperature was 26°C.

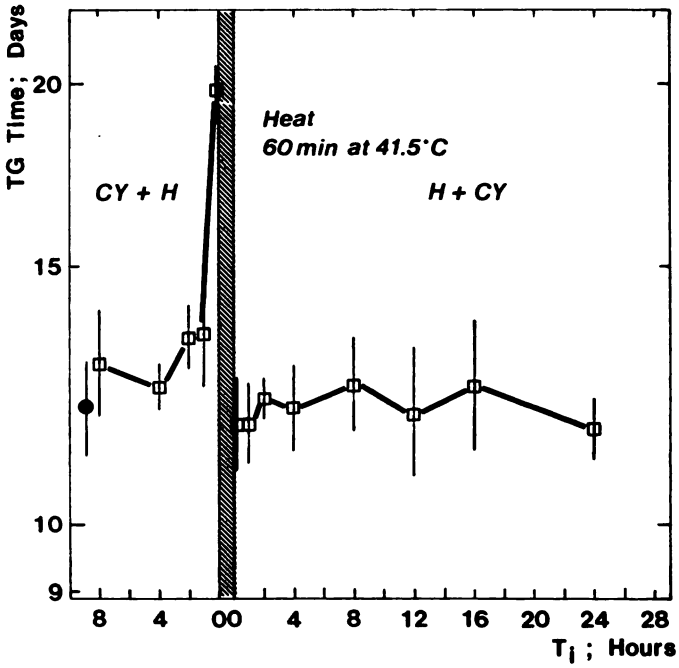

Chart 3. The effect of treatment intervals between CY and heat (*H*) and between heat and CY on the TG time. A CY dose was 100 mg/kg, and hyperthermia was 60 min at 41.5°C. ●, TG time following a CY dose of 100 mg/kg administered without heating. *Bars*, 95% confidence limits. T_I, time interval between treatments.

40.5 and 41.5°C, the effect of CY was enhanced at these temperatures. CY alone prolonged the TG time approximately 3 days compared to the nontreated animals, as shown at 0-min treatment time. The enhancement increased with increasing treatment time, but it reached plateau after ≃20 min of hyperthermia. Above 42.5°C, hyperthermia alone prolonged the TG time. Therefore, the TG time following thermochemotherapy was continuously prolonged with increasing treatment time. This indicates that thermochemotherapy showed an additive effect together with thermal enhancement of the chemotherapeutic effect at these temperatures. To compare the size of the enhancement at different temperatures, the TG time following a 20-min treatment was plotted as a function of the treatment temperature (Chart 2). The ratio of the TG time (CY and heat) to the TG time (heat alone) was independent of temperature at 40.5°–44.5°C. Similarly, the ratio of the TG time (glucose, CY, and heat) to the TG time (heat alone) was constant in the same temperature range.

Although a time interval of 30 min between the CY administration and the beginning of hyperthermia has been used in our study, a recent report indicated a relatively rapid clearance rate of the active form CY from mouse plasma (13). Accordingly the effect of the time interval between CY administration and hyperthermia on the TG time was investigated (Chart 3). The CY dose was 100 mg/kg, and hyperthermia was 60 min at 41.5°C. Notably the CY given immediately before the beginning of hyperthermia was the most effective as evidenced by a substantial prolongation of the TG time. The CY administered 1 to 8 h before hyperthermia was slightly more effective than the CY alone. Hyperthermia given before CY administration did not enhance

the effect of CY. Based on these results, the effect of CY was reinvestigated at various temperatures by administering it immediately before heating. The CY dose was reduced to 100 mg/kg, since this dose given immediately before 41.5°C hyperthermia was slightly more effective than a dose of 200 mg/kg administered 30 min before 41.5°C heating.

The TG time following thermochemotherapy prolonged with increasing treatment time at any temperature tested (Chart 4). At temperatures below 42.5°C which did not or only slightly prolonged the TG time by heat alone, the effect of CY was enhanced substantially. This enhancement tends to increase rapidly in the first 30 min and then to approach a plateau. At 43.5°C the enhancement was less extensive compared to temperatures at 40.5°–42.5°C, although an additive effect of hyperthermia was observed. The findings at 44.5°C were similar to those at 43.5°C, except that the enhancement was rather insignificant. To compare the size of enhancement at different temperatures, the TG time following a 20-min treatment was shown as a function of treatment temperature (Chart 5). These results indicate that combined CY and hyperthermia prolongs the TG time most effectively at temperatures of 41.5°–42.5°C. A similar comparison for a 60-min treatment indicated that 40.5°C was almost as equally effective as 41.5°C.

Another interesting observation in this series of experiments was that hyperglycemia did not further enhance the effect of CY.

The next experiment investigated the effect of thermochemotherapy as a function of CY dose at various temperatures. Hyperthermia was for 30 min at each elevated temperature, and the CY was given immediately before hyperthermia. The TG time prolonged with increasing CY dose at any temperature tested (Chart 6). The prolongation was most substantial at temperatures of 40.5°–42.5°C. The effect was also investigated in a 37°C water bath, since the tumor temperature was 25°–26°C when animals were kept in room temperature. The effect of 37°C was between the effect of room temperature and that of 40.5°C. The CY dose response curves at temperatures above 37.0°C were downward concave. This may indicate that some fractions of tumor cells were resistant to combined CY and heat treatments or that the effect of CY was saturated at high drug concentrations.

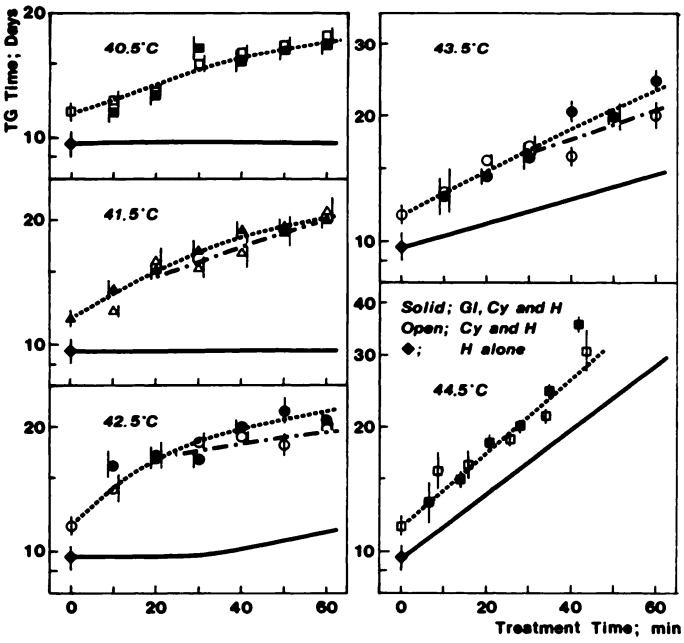

Chart 4. The effect of thermochemotherapy with or without glucose (*GI*) on the TG time of the FSa-II tumor. CY of 100 mg/kg was given i.p. immediately before hyperthermia, while glucose of 5 mg/g was given 60 min before hyperthermia (*H*). *Solid* and *open symbols* indicate that thermochemotherapy was given with and without glucose, respectively. Dose response curves for hyperthermia alone were taken from Ref. 22 and normalized. *Bars*, 95% confidence limits.

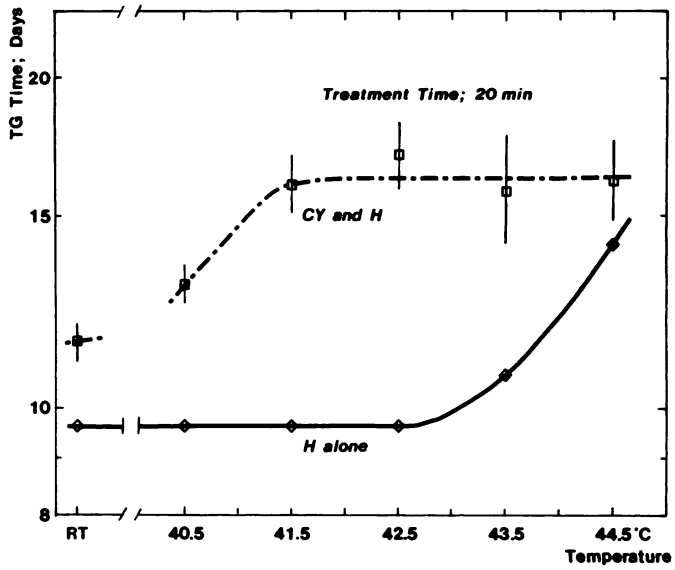

Chart 5. Comparison of the effect of thermochemotherapy at various temperatures. All data are taken from Chart 4. The ratio of the TG time [CY and heat (*H*)] to the TG time (heat alone) is largest at 41.5°C and 42.5°C. *Bars*, 95% confidence limits.

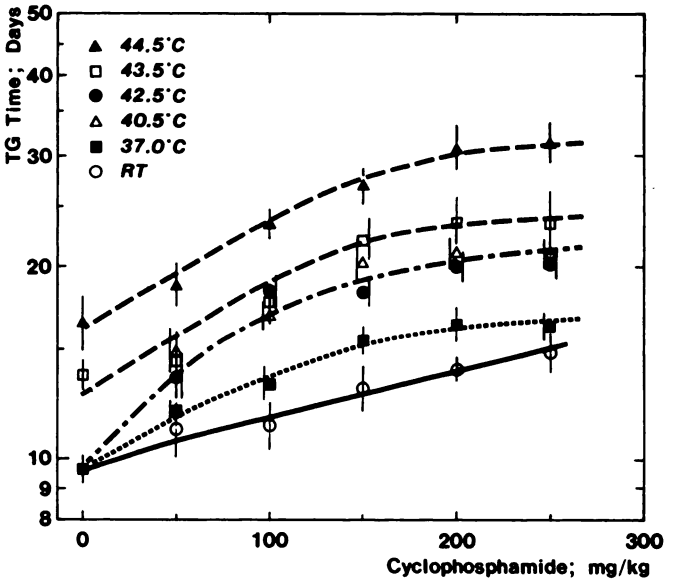

Chart 6. Dose response curves for the FSa-II tumors following thermochemotherapy given at various temperatures. A CY dose was given immediately before hyperthermia. The duration of heat treatment was 30 min at each temperature. *RT* indicates treatment at room temperature without immersing the tumor into a water bath. *Bars*, 95% confidence limits.

DISCUSSION

The present results can be summarized as follows. (*a*) Thermal enhancement of the cytotoxic effect of CY was independent of

temperatures above 40.5°C when the CY was given 30 min before hyperthermia. (*b*) Combined CY plus hyperthermia was most effective at temperatures of 40.5°–42.5°C when the CY was given immediately before hyperthermia. (*c*) The effect of CY was most substantially enhanced when the CY was given immediately before hyperthermia. (*d*) Hyperglycemia enhanced the effect of thermochemotherapy when the CY was administered 30 min before heat, but this enhancement was eliminated when the CY was given immediately before hyperthermia. (*e*) The CY dose response curves at temperatures above 37.0°C were downward concave.

Various mechanisms have been proposed for thermal enhancement of the cytotoxic effect of various types of chemotherapeutic agents (1). For the alkylating agents, Johnson and Pavelec (3) suggested that the rate constant for inactivation increased not only with drug concentration, but also with increasing temperature. The inactivation energy found on the Arrhenius plot was compatible with that of the alkylation reaction (≃35 kcal/M) at the temperature below ≃42.0°C. Above 42.0°C, the inactivation energy for combined alkylating agent and heat treatments was the intermediate between the inactivation energy of alkylation and that of thermal denaturation (≃140 kcal/M). Above 44.0°C, however, thermal denaturation became the dominant reaction over alkylation. These observations are apparently compatible with the present result that thermal enhancement of CY, an alkylating agent, was the most substantial at temperatures of 40.5°–42.5°C. This is quite attractive for clinical applications of thermochemotherapy, particularly with alkylating agents, since higher temperatures may induce intolerable side effects which may require the termination of hyperthermia. The presence of a cold spot in the tumor, which is inevitable by present hyperthermia technology, may be overcome by the simultaneous or preheating administration of an alkylating agent. It should be noticed that the effective temperature of thermochemotherapy depends on the chemotherapeutic agent or the type of chemotherapeutic agents. The effect of some agents is enhanced at temperatures above 43.0°C, while the effect of most alkylating agents is well enhanced below 43.0°C (1, 2). Another advantage of thermochemotherapy might be that local thermochemotherapy may enhance the local tumor response without enhancing the systemic side effect or the side effects observed in other organs which are not in the treatment field.

Our previous paper described that hyperglycemia given 60 min before hyperthermia enhanced the effect of CY at an elevated temperature (6). In the first experiment of the present study this phenomenon was also observed (Chart 1), since the CY was given 30 min before the beginning of hyperthermia as in the previous study. This enhancement was not observed when the CY was administered immediately before hyperthermia. This observation raised a question about the mechanism of hyperglycemia enhancement. Previously the decrease in the tissue pH was considered to be the major mechanism. If this is the only mechanism, the enhancement should have been observed when the CY was given immediately before heat. Besides decreasing tissue pH, hyperglycemia increases osmotic pressure of the extracellular fluid and blood viscosity and decreases blood flow (15, 16). The reduced blood flow may result in a longer plasma or tissue half-life of the drug. An i.p. administration of mannitol, which also increases the osmotic pressure without altering blood and tissue pH, was able to enhance the thermal response of the FSa-II tumor.[6] Accordingly the enhancement by hyperglycemia might be attributed to the dual mechanism, *i.e.*, a decrease in the tissue pH and a decrease in the blood flow. Lack of hyperglycemia enhancement observed following CY administration given immediately before hyperthermia may be due to the reduced blood flow with a resultant decrease in the drug uptake into the tumor. Further studies are needed to investigate the mechanism of the enhancement and the pharmacokinetics of the drug in hyperglycemic animals.

A difference observed in the thermal enhancement of the CY given 30 min before and immediately before hyperthermia at 43.5°C and 44.5°C (compare Charts 1 and 4) may be due to the different concentration of the active form CY at the time of hyperthermia. Cyclophosphamide must be activated *in vivo*, and this active form CY may be available in greater amounts in the tumor during the 30-min treatment period if hyperthermia was given immediately after CY injection, compared to the same treatment period beginning 30 min after CY administration. The effect of combined CY and heat treatments on a solid tumor may be regulated not only by the reaction rate at a given temperature but also by the rate of formation of active products (14) and the drug delivery mechanism, particularly by the blood flow rate (17) which is strongly influenced by the temperature.

The shape of CY dose response curves was downward concave at temperatures above 37.0°C. This may indicate the presence or the development of drug-resistant cell fractions, the saturation of drug effect at high drug concentrations, or the limited capability of the host to produce the active form CY. The downward concave shape may be due to mutation, selection, specific tumor physiology, hyperthermia itself, or induced drug-resistance (18). Morgan *et al.* (19) found that preheated tumor cells which acquired thermal resistance were also resistant to bleomycin and 1,3-bis(2-chloroethyl)-1-nitrosourea. This is an important observation but does not explain the present results which were observed during a single heat treatment with a large dose of CY. The tumor might have contained a CY-resistant cell population. The tumor cells might have been saturated with the drug after an administration of a large CY dose as has been suggested following an administration of a large dose of bleomycin (20, 21). Although the mechanism of the downward concave shape must be reserved for further investigation, this phenomenon could limit the effect of thermochemotherapy.

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