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Effect of Hyperthermia and/or Nicotinamide on the Radiation Response of a C3H Mammary Carcinoma

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Abstract—*The effect of hyperthermia and/or nicotinamide (200 mg/kg of body weight) on the tumour growth delay induced by radiation was evaluated in a C3H mouse mammary adenocarcinoma. The study showed a radiosensitizing effect of hyperthermia and of nicotinamide but the combination of all three modalitites showed no increased tumour growth delay compared to hyperthermia and radiation alone. The tumour growth delay induced by hyperthermia was not modified by nicotinamide.*

INTRODUCTION

NICOTINAMIDE and hyperthermia are both known to enhance the cytotoxic effects of ionizing radiation. Using the tumour growth assay, we have previously reported a dose modification factor of 1.2 when nicotinamide was combined with radiation in the treatment of C3H mammary adenocarcinoma [1]. Similar levels of enhancement have also been reported for other tumour systems [2]. At present some confusion exists as to the mechanism of radiosensitization by nicotinamide, both repair inhibition and increased oxygenation have been implicated [3, 4]. However, its low toxicity in man [5–7] makes it an attractive possibility for clinical development. Hawkins [5] gave nicotinamide daily in doses of up to 12 g when treating patients with schizophrenia, although some patients developed gastrointestinal flu-like symptoms at this high dose. According to Ranchoff and Tomecki [7], doses of up to 6 g/day of nicotinamide are safe.

Nicotinamide is an inhibitor of the chromatin-bound enzyme adenosine diphosphate ribosyl transferase (ADPRT) and ADPRT has been linked to the mechanism of DNA repair after radiation damage [3]. Upon activation, by introducing nicks in the DNA, ADPRT catalyses the cleavage of NAD^+ to ADP-ribose and nicotinamide. Thus it is known that the cellular pool of NAD^+ drops after DNA damage has been introduced into cells [8, 9]. The NAD pool can, however, be restored after radiation [8]. We have previously shown that hyperthermia will inhibit this restoration of the NAD pool [10]. This persisting NAD depletion can then act as an indirect inhibitor to ADPRT and thus interfere with the repair of DNA damage. It has also been shown that the metabolism of ADP-ribose(n) is inhibited by hyperthermia and that the enzyme involved in its degradation, poly(ADP-ribose) glycohydrolase, is inactivated by heat shock [11].

Reports in the literature indicate that ADPRT inhibitors can also increase the *in vitro* cytotoxicity of hyperthermia, whether applied alone or in combination with radiation [12, 13]. One of these studies [13] indicates a similar action of heat and ADPRT inhibitors (m-aminobenzamide). An *in vivo* study has also shown an increased cytotoxicity in mouse tumours when nicotinamide (1000 mg/kg) was combined with heat and radiation [14].

The aim of this study was to investigate the effects of a clinically relevant dose of nicotinamide on the radiation response and heat response of a C3H mouse adenocarcinoma *in vivo*. We have also investigated the combined effect of heat and nicotinamide on irradiated tumours. Tumour growth delay has been used for evaluation of response.

MATERIALS AND METHODS

Mice and tumours

C3Htif/bom mice of both sexes were used when they were 6–8 weeks of age. A viable piece of tumour tissue was taken from a donor mouse, minced with a pair of scissors and pressed through a syringe needle (gauge 23). When the tumour passed through the needle with ease, 0.1 ml of the

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tumour brei was inoculated subcutaneously into the left hind foot of each mouse. Tumours were selected for treatment approximately 11 days later when they were 4.9 ± 0.7 mm in diameter. Following treatment, tumours were measured three times weekly and the animals were sacrificed when the tumours had reached five times their starting volume.

The experiment was performed over the period of 1 year. Four to five control animals were incorporated into each treatment batch to check that tumour growth characteristics had not changed during the experimental time.

For each animal the time was determined for the tumour to regrow to 3.5 mm larger than the size at treatment. The mean regrowth time could then be calculated for each group.

Animals which survived 90 days without evidence of tumour regrowth were allocated a tumour growth time equal to the longest time recorded for any treated animal (125 days). Tumours recurring at such late times were visible at day 70. Dose groups containing these locally controlled animals are indicated by an upward arrow attached to the error bar (±1 S.E.M.) in Figs. 1–4. No difference in tumour growth could be detected in male and female mice.

Nicotinamide treatment

Nicotinamide (Sigma) was dissolved in saline and based on previous studies, was injected i.p. at a dose of 200 mg/kg body weight 30 min before the start of the heat or radiation treatment.

Hyperthermia treatment

Hyperthermia was administered using a temperature controlled water bath heating system. The water temperature was set at 0.3°C higher than the desired tumour temperature [15]. Hyperthermia treatments were applied immediately before irradiation and lasted for 30 min.

Radiation treatment

A linear accelerator (Philips SL75/14) producing 8 MV X-rays and provided with a secondary field forming lead collimator (at a distance of 70 cm from the focus) was used for irradiation of the tumours. Non-anaesthetized animals were fixed in plexiglas holders with the tumour-bearing leg stretched out in the radiation field and immersed in a small water phantom acting as a bolus. Groups of five animals were treated with the tumour positioned at dose maximum in the beam. Absolute dosimetry and tests of field flatness were performed with ionization chamber and confirmed with thermoluminescent dosimetry measurements. The dose variation between the five positions in the radiation beam was less than ±3%. The dose rate was 4 Gy/min.

RESULTS

Nicotinamide combined with radiation

Figure 1 shows both the growth curves and the dose–response relationship for tumours treated with a single dose of radiation applied either alone or 30 min after 200 mg/kg nicotinamide. In each instance, nicotinamide increased the delay in tumour regrowth produced by radiation alone. The difference, however, is only significant at 15 Gy ($P = 0.046$, Student's t-test).

Nicotinamide combined with heat

Tumour data for combined treatment with heat and nicotinamide are shown in Fig. 2. Heat alone had a significant effect on tumour growth for all temperatures above 41.0°C ($P = 0.041$, $P = 0.024$, $P = 0.006$). By contrast, treatment with 41°C for 30 min actually *increased* the rate of tumour growth compared to controls ($P = 0.003$). When nicotinamide was injected 30 min before heat no significant increase in growth delay was measured. At the highest temperature tested, 43°C for 30 min, local tumour control was achieved in four animals treated with heat alone, and three treated with heat plus nicotinamide. It was thus concluded that nicotinamide (200 mg/kg) had no effect on the response of tumours to hyperthermia.

Radiation combined with heat

In Fig. 3, data for tumours treated with radiation with or without heat are shown. The heat treatment alone (42°C for 30 min) had a small but significant ($P = 0.041$) effect on tumour growth. This gave rise to a significant enhancement of the radiation induced growth delay at 7.5 Gy ($P = 0.002$); higher radiation doses were not tested with this dose of heat. However, when 43°C for 30 min was combined with 15 Gy, all of the tumours were cured (data not shown). These data indicate a considerable sensitizing effect of moderate hyperthermia on this tumour.

Radiation, heat and nicotinamide

The results for the combined treatment of radiation (7.5 Gy), heat (42°C/30 min) and nicotinamide are shown in Fig. 4. As indicated in the previous figures, heat was a much better radiosensitizer than nicotinamide. When the three treatments were combined, the response was indistinguishable from that for radiation and heat alone ($P = 0.884$). There was also no difference in the number of tumour cures between the heat and X-ray group (1/15 cured) and the heat, radiation and nicotinamide group (2/15 cured).

DISCUSSION

The *in vivo* enhancement of radiation damage by hyperthermia has been well established [16].

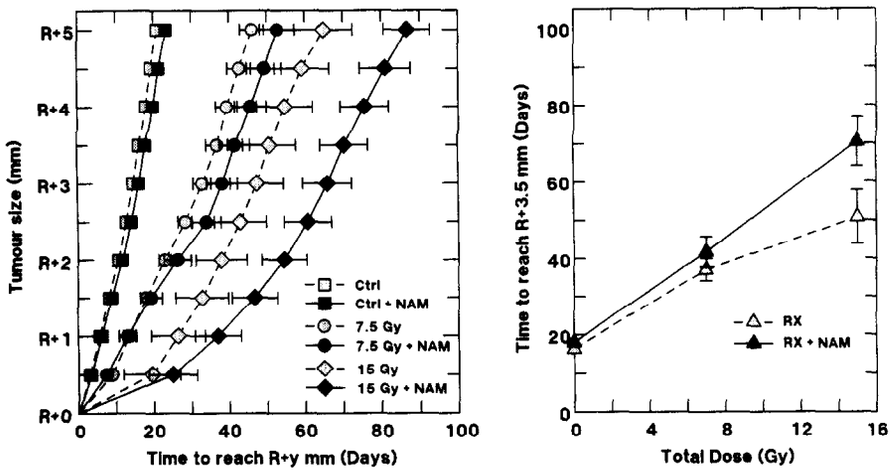

Fig. 1. *Growth curves and dose–response relationships for tumours treated with a single dose of radiation applied either alone or* 30 *min after* 200 *mg/kg nicotinamide. R = treatment size. Bars represent* 1 *S.E.M.*

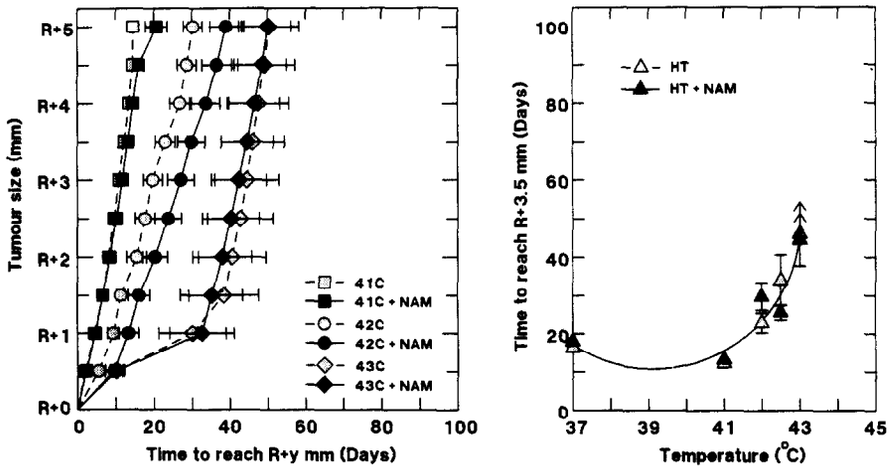

Fig. 2. *Growth curves and temperature response relationship for tumours treated with hyperthermia applied either alone or with nicotinamide. R = treatment size. Bars represent* 1 *S.E.M.*

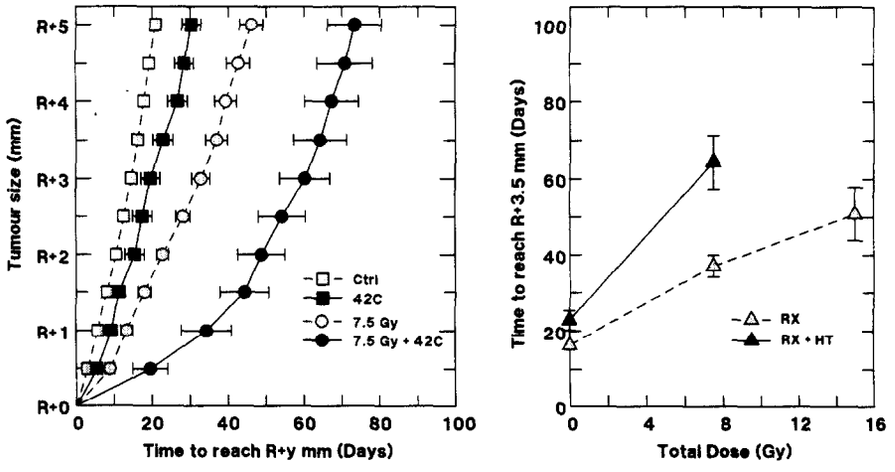

Fig. 3. *Growth curves and dose–response relationship for tumours treated with radiation alone or radiation and heat. R = treatment size. Bars represent* 1 *S.E.M.*

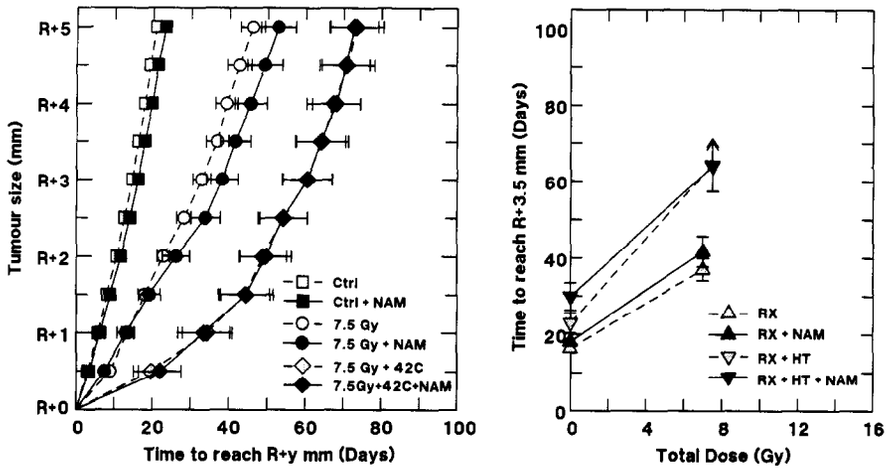

Fig. 4. Growth curves and dose–response relationship for tumours treated with radiation alone, radiation and nicotinamide, and radiation, nicotinamide and heat. R = treatment size. Bars represent 1 S.E.M.

Likewise, several reports have described the radiosensitization which can be achieved with nicotinamide [1, 2, 17, 18]. Since analogues of nicotinamide have also been shown to increase thermal toxicity *in vitro*, we decided to investigate this combination *in vivo* and also to test whether nicotinamide could further increase the effectiveness of a combined heat and radiation treatment.

The data presented show that a moderate dose of hyperthermia, 42°C for 30 min, can itself have an effect on tumour growth as well as significantly increasing the growth delay achieved with radiation. Since only one dose of radiation was used, it is not possible to quantitate the degree of sensitization in terms of a thermal enhancement ratio. As previously demonstrated for this tumour [1], a low dose of nicotinamide (200 mg/kg) can also produce radiosensitization. In the present study, only at 15 Gy did the combined treatment result in a significantly increased growth delay and it is apparent that, at the doses used, heat is much more effective than nicotinamide at potentiating the tumour response to radiation.

When nicotinamide was combined with hyperthermia, at no temperature was the tumour response significantly enhanced (Fig. 2). This is in contrast to the *in vitro* data where ADPRT inhibitors have been found to increase the cell killing effects of hyperthermia, particularly when the cells were exposed to the drug both during and after the heat treatment [13]. Although we injected the drug 30 min before hyperthermia commenced, its plasma half-life of 84 min (measured by HPLC), would ensure its presence in high concentration both at the time of heating and for at least 1 h afterwards. The drug dose chosen may have been insufficient to significantly inhibit poly(ADP-ribose) transferase. However, it did produce sensitization of radiation damage especially at the higher of the two X-ray doses tested. This might indicate that, as suggested by Horsman *et al.* [4], the main effect of nicotinamide *in vivo* is to improve tumour oxygenation via an increase in blood flow. If this were so, it is not surprising that thermal sensitization was not achieved, since the cells most sensitive to heat are those under conditions of oxygen and nutrient deprivation [19].

The data of Horsman *et al.* [4] show that 1000 mg/kg of nicotinamide can reduce the tumour binding of radiolabelled misonidazole, which is considered a good indicator of tumour hypoxia. They have also measured significant increases in tumour blood flow. Using a different tumour system, the CaNT in CBA mice, we have recently confirmed these findings. Using ^{86}RbCl extraction we have measured a greater than 50% increase in tumour blood flow which persists for at least 2 h after injection and is indistinguishable for doses of either 500 or 1000 mg/kg of drug (unpublished data). However, when we examined the dose used in the present investigation (200 mg/kg body weight), no effect on tumour bloodflow could be detected. Thus, although the radiosensitization achieved with high dose nicotinamide may involve improved tumour oxygenation, this seems unlikely to explain the effects measured with low doses of drug.

We have measured no enhancement by nicotinamide of the tumour response to combined hyperthermia and radiation, although both heat and nicotinamide produced radiosensitization in this system. This is in contrast with the report of Horsman *et al.* [14] for a mammary carcinoma in C3H/Tif mice, tessted by local tumour control. In a previous study *in vitro*, we have shown an additive toxicity of heat, nicotinamide and radiation, towards human mononuclear leukocytes [20]. Similar data exist for V79 cells, where the combination of heat, an ADPRT inhibitor and radiation produced greater

cytotoxicity than heat and radiation alone [13]. The heat dose chosen in the current study produced significant additional delay when used alone (7 days), or in combination with 7.5 Gy (48 days). This large thermal sensitizing effect may have masked any small degree of radiosensitization induced by nicotinamide. However, since 7.5 Gy was the only radiation dose tested with the three-agent combination, a greater range of doses of each agent should be tested before firm conclusions are drawn. Despite the negative results of the three-agent schedule, this study does not emphasize that radiosensitization can be achieved by low, clinically achievable doses of nicotinamide. The mechanism involved remains unclear.

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