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Effects of Cytostatic Drugs and 40.5°C Hyperthermia on Human Clonogenic Tumor Cells*

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Abstract—*A tumor colony-forming assay was used to investigate the effect of* 40.5°C *hyperthermia and drugs on the colony-forming ability of human clonogenic tumor cells. In order to be able to perform repeated incubations with identical tumor material, specimens were used that were augmented in the nude mouse system. Five tumor samples (two malignant melanomas, two squamous cell carcinomas of the lung and one small cell carcinoma of the lung) were incubated with seven drugs: doxorubicin, actinomycin-D, bleomycin, melphalan, vincristine, vinblastin and cisplatinum. One additional tumor specimen (chondrosarcoma) was incubated with doxorubicin immediately after resection. The incubation with drugs at* 37°C *revealed dose response curves typical for each tumor. Incubation at* 40.5°C *for* 2 hr *showed enhanced drug effects in five out of the six tumors tested. The drugs, with an enhanced effect and the pattern of enhancement were different in each individual tumor.*

INTRODUCTION

IT HAS been reported that hyperthermia is able to enhance the effect of many cytostatic drugs [1].

Hahn was able to demonstrate for one group of cytostatic drugs that a linear reduction of cell growth can be obtained when the temperature is raised from 37 to 43°C. Other drugs showed threshold effects, i.e. an enhancement was only observed when the temperature was above 42°C [2]. In the treatment of disseminated malignancies attempts have been made to combine whole-body hyperthermia with cytostatic drugs [3–6]. In this modality the temperature level must be restricted to a tolerable range, i.e. below 42°C. The aim of this study was to find out whether the effects of cytostatic drugs can be enhanced by a moderate hyperthermia of 40.5°C for 2 hr.

Most of the basic *in vitro* experiments concerning the combination of hyperthermia and cytostatic drugs were performed with animal cell lines [7]. In this study we used tumor cells from human spontaneous tumors cultured in a tumor colony-forming assay.

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MATERIALS AND METHODS

Freshly resected human tumor material is usually available only in small amounts and cannot be used for repeated experiments. We therefore implanted the tumor samples in the nude mouse system, in order to have more homogeneous tumor tissue for repeated experiments. Five different human tumors were used. The histologic types were: amelanotic melanoma, small carcinoma of the lung and two squamous cell carcinomas of the lung. Another tumor sample from a chondrosarcoma was used immediately after resection. Only one drug could be tested in this case.

Culture assay for clonogenic human tumor cells

Tumor cells were cultured as described elsewhere [8]. Briefly, tumor samples were cut into small pieces by a scalpel after having removed collagenous tissue. A single-cell suspension was obtained by repeated aspiration into a syringe through a gauge needle No. 20. This procedure was performed in Iscove's modified Dulbecco's medium (IMDM) (Gibco) containing 10% FCS (Boehringer Mannheim). Viable cells 1×10^5 were plated in the presence of 30% FCS in IMDM. The viability of the cells was assessed by trypan blue exclusion. Methylcellulose (final

concentration 0.9%) was used as a viscous support.

Cells were incubated at a CO_2 concentration of 7.5%. The pH in the dishes was 7.4 in both the normothermic and the hyperthermic cultures. Normothermic dishes were placed in an incubator at a temperature of 37°C. The temperature was monitored by an electronic thermosensor in a sham culture dish containing the same amount of methylcellulose and serum as the dishes containing tumor cells. The variance of the temperature was ±0.1°C.

Hyperthermic cultures were exposed to heat for 2 hr at 40.5°C in an incubator. Afterwards the culture dishes were placed in a normothermic incubator. After further incubation for 8–10 days, colonies were counted under an inverted microscope. Drug effects were expressed as percent survival of tumor cell colonies as a function of drug concentrations as outlined by Salmon and co-workers [9].

Colonies were considered as cell aggregates if of more than 30–40 cells, with a diameter of 80–100 μm or more. The tumor colonies were ball-shaped and translucent.

Drugs

The tumor cells were incubated with the cytostatic agents doxorubicin (Farmitalia), actinomycin-D (Sharp & Dome), bleomycin (Mack), cisplatin (Bristol), vincristine (Lilly), vinblastine (Lilly) and melphalan (Wellcome). The drugs were diluted with 0.9% NaCl solution or distilled water and added to the culture medium, so that the tumor cells were exposed to the drugs continuously. Drug dose concentrations were chosen over a 2–3 log concentration range as proposed by Salmon *et al.* [9].

RESULTS

Cells from five individual human tumors grown in the nude mouse system and from one tumor sample that was resected freshly from a tumor were plated in a methylcellulose monolayer system. The cultures were incubated with different cytostatic drugs at 37°C and for 2 hr at 40.5°C. This short period was chosen in order to test an exposure time that is achievable *in vivo* for eventual treatment of patients. The plating efficiency of the tumor cells is shown in Fig. 1. The assay proved to be well reproducible. At 40.5°C no cell killing is observed. In some cultures the plating efficiency was increased compared to the normothermic control dishes, as can be seen in Figs. 2–7, but this difference was not statistically significant. The incubation with drugs at different concentrations showed individual dose response curves typical for each tumor and each drug.

In the amelanotic melanoma XF 236 (Fig. 2) colony reduction was enhanced under hyperthermic conditions when incubated with actinomycin-D, melphalan, vincristine, bleomycin and, to a small degree, vinblastine. In the malignant melanoma XF 462 (Fig. 3), however, the effects of vincristine, melphalan and bleomycin were enhanced. In the case of cisplatin

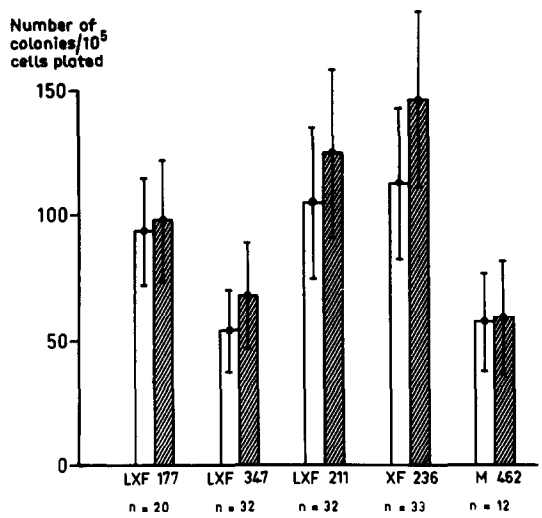

Fig. 1. The numbers of colonies from five different human tumors per 1×10^5 *cells seeded. The blank columns indicate the normothermic, the shaded columns the hyperthermic cultures without cytostatic drugs. The number of culture dishes evaluated for normothermic and hyperthermic cultures is indicated at the bottom of the columns. The bars represent the standard deviation. The* 40.5°C *hyperthermic incubation was performed for* 2 hr.

at 1.0 μg/ml in normal and hyperthermic incubation no colony formation was observed. It is therefore not possible to state whether an enhancement of the drug effect occurred or not.

In the squamous cell carcinoma of the lung XF 347 (Fig. 4) only a small enhancement could be demonstrated under incubation with actinomycin-D.

The squamous cell carcinoma of the lung LXF 211 (Fig. 5) did not show any difference between normothermic and hyperthermic cultures in all drugs tested.

The small cell carcinoma of the lung, XF 177 (Fig. 6), showed a slightly decreased colony formation with hyperthermia when incubated with vincristine. The chondrosarcoma cells were incubated only with doxorubicin. In this case a strong hyperthermic enhancement of the drug effect was seen (Fig. 7). We did not use higher temperatures because in this assay temperatures above 41°C and longer incubation periods induced an increase of lactic acid in the culture

dishes which resulted in a cell kill by acidification. At 40.5°C for 2 hr no difference in pH between normothermic and hyperthermic cultures was observed.

DISCUSSION

Data from *in vitro* and *in vivo* experiments investigating the combination of heat and cytotoxic drugs at temperatures above 42°C are important with respect to local hyperthermia. With regard to whole-body hyperthermia, the temperature must be kept below 42°C. The aim of this study was to find out if an enhancement of cytotoxic drug effects can be obtained in the low hyperthermic range compatible with whole-body hyperthermia conditions. The clonogenic assay

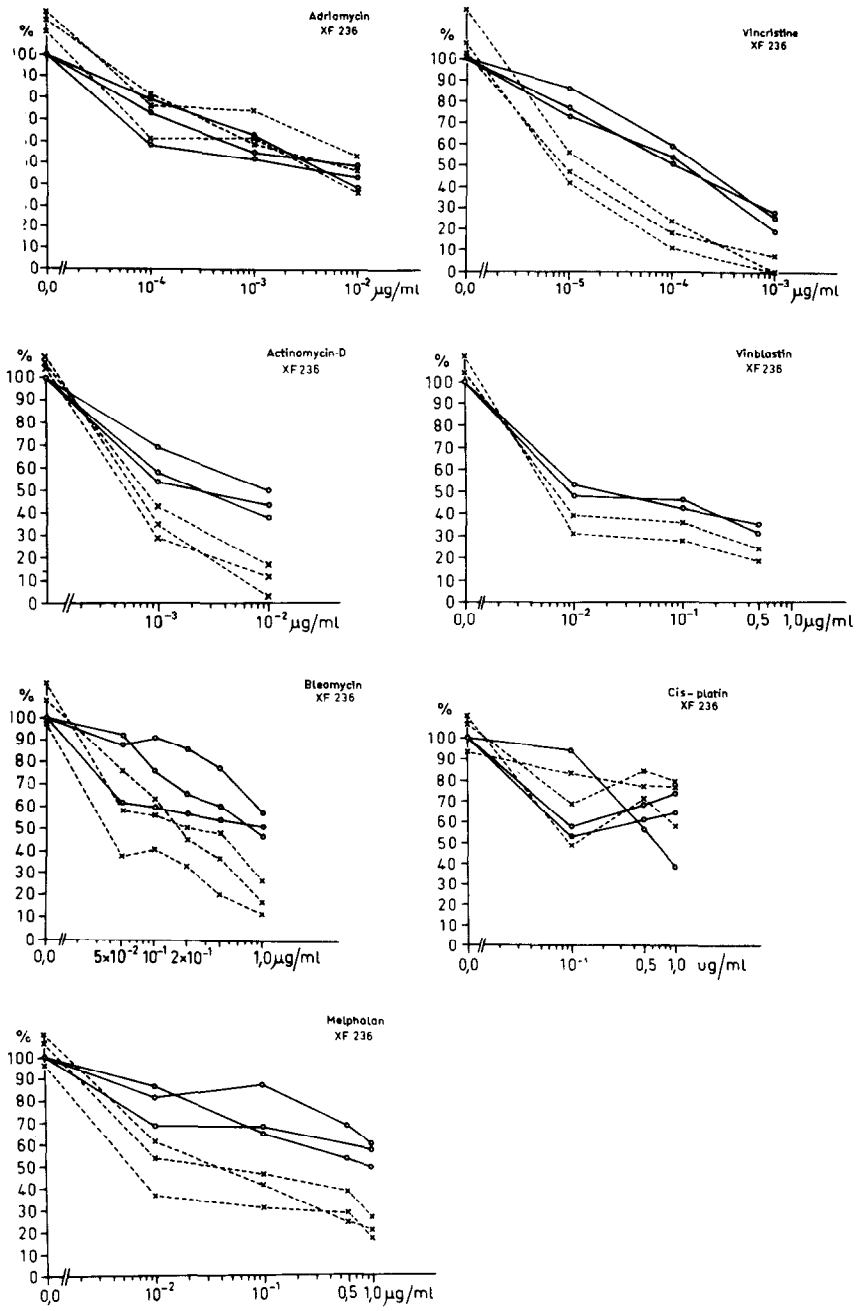

Fig. 2.

Figs. 2–7. Dose response curves of human tumor colonies under incubation with several cytostatic drugs (indicated in the chart). Untreated controls are set as 100%. *The drug effect is expressed as % survival of colonies as a function of drug concentration (abscissa). MM* 462, *XF* 236 = *malignant melanoma; LXF* 347, *LXF* 211 = *squamous cell carcinoma of the lung; LXF* 177 = *small cell carcinoma of the lung. The chondrosarcoma was seeded immediately after resection. The normothermic cultures are indicated by the open circles and the solid lines, the hyperthermic cultures by the* × *and the dotted lines.*

for human spontaneous tumor cells provides the possibility to test the drug sensitivity of human tumors individually. The results from this assay are expected to be clinically more relevant than those obtained from experimental cell lines. Salmon *et al.* could demonstrate [10] that a clonogenic assay can predict a resistance of a tumor to chemotherapy in 95% of the cases and a sensitivity in 61% of the tumors tested. The experiments from Mann *et al.* [11] using a human tumor colony-forming assay with drugs and hyperthermia were regarded as encouraging. They reported that 4/5 patients with malignant melanoma showed clinical response to thermochemotherapy, as had been predicted in the *in vitro* assay.

In our experiments the effect of doxorubicin was not enhanced in five tumors at 40.5°C. Only in the chondrosarcoma did a dramatic enhancement occur under hyperthermia (Fig. 7). These results are partly in agreement with Hahn's data, which described doxorubicin as a drug that is not or only minimally enhanced at temperatures below 42°C [2, 7]. On the other hand, animal experiments performed by Overgaard [12] demonstrated that the combination of doxo-

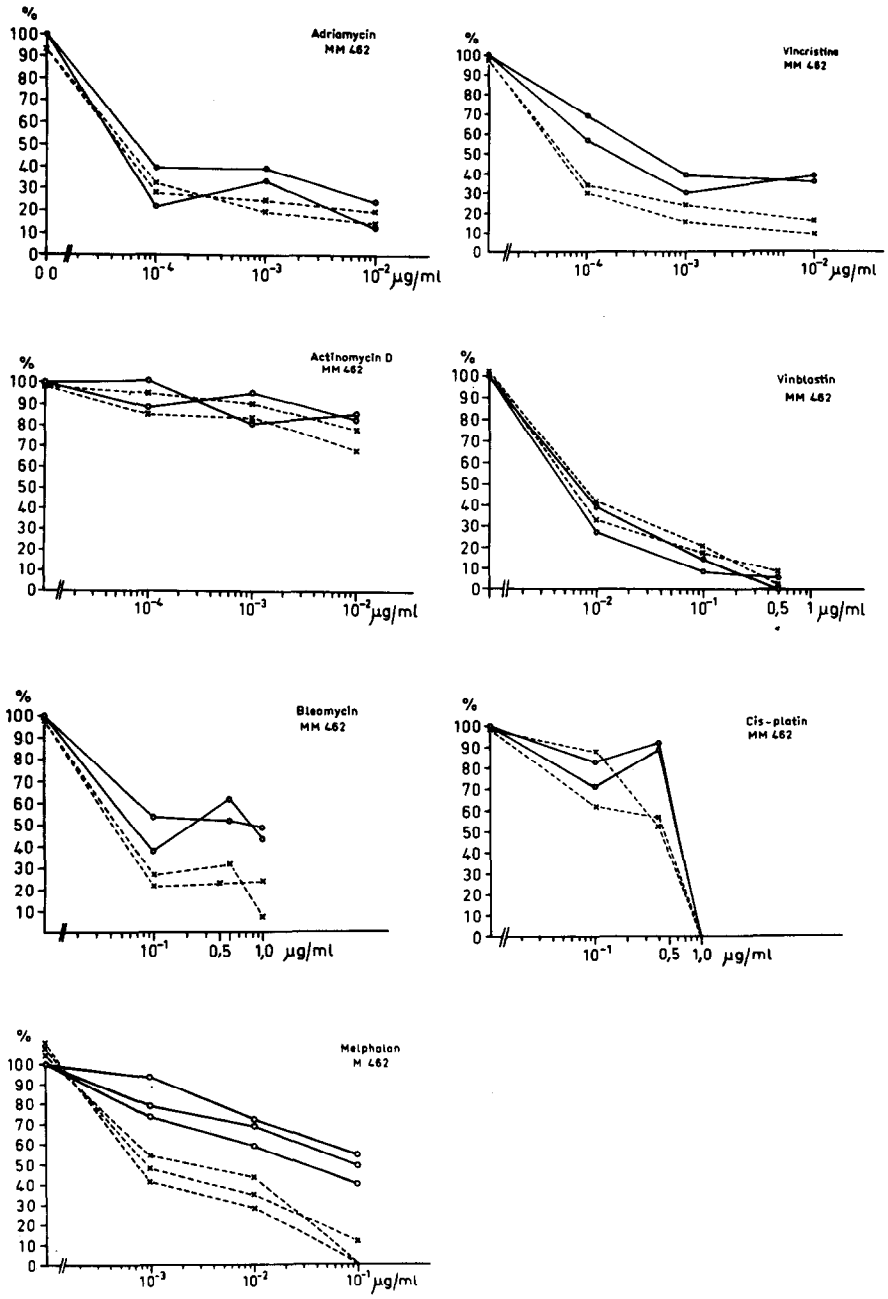

Fig. 3.

rubicin and hyperthermia (40.5 and 42.5°C) for 120 min resulted in complete regressions in most of the tumors. Whole-body hyperthermia at 40.5°C showed a less distinct response than local hyperthermia.

But these different results must be seen in context with the different test-systems. While Hahn [1, 2] performed his experiments mostly with CHO-cell lines, Overgaard [12] used a transplantable mammary carcinoma. Both systems are different from the system used in our experiments.

Dahl [13] has studied the effect of doxorubicin and hyperthermia on a neurogenic rat line *in vitro*. He found a synergism at 42°C. At 41°C he found a biphasic response with an initial sensitive phase followed by a more resistant one. Like Hahn [14], he suggested that the cell became tolerant to hyperthermia at longer exposition to hyperthermia.

Bleomycin is described as a drug that is not enhanced by low hyperthermia [2, 15]. In our experiments colony reduction was enhanced at 40.5°C in the two malignant melanomas. Like in some other tumors, in the melanoma 462 in both the normothermic and the hyperthermic cultures

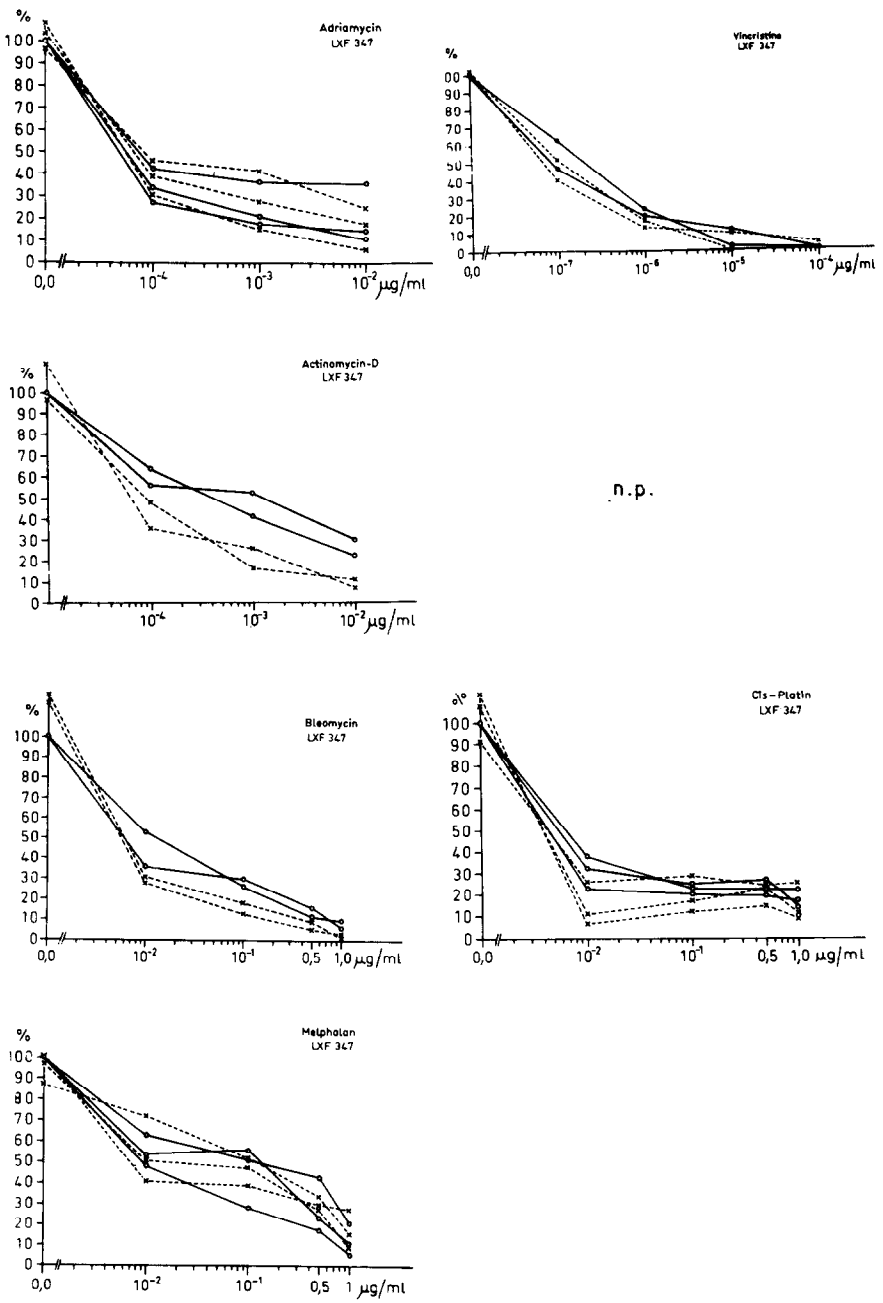

Fig. 4.

after a rapid decrease of colony formation a plateau was found at higher drug concentrations, suggesting a resistant subpopulation of tumor cells.

Actinomycin-D is another 'threshold drug' [2]. Several papers report a synergistic killing of actinomycin-D and hyperthermia at temperatures above 42°C [1, 15]. A protective effect of hyperthermia against actinomycin-D, as had been described by Donaldson *et al.* [16] at 43°C and long-term hyperthermic exposition, was not observed under our conditions. We observed synergistic effects in the amelanotic melanoma and, to a smaller degree, in the squamous cell carcinoma of the lung.

Melphalan is a drug that is successfully used for regional perfusion of the limbs in the treatment of malignant melanoma patients [17–19]. Goss and Parsons [20] compared the effect of hyperthermia and melphalan on seven melanoma lines and embryonic melanoma cells. Two out of seven cell lines were heat-sensitive. In one melphalan-sensitive line at 40°C a 10-fold cell kill was observed under hyperthermic conditions. In the two malignant melanomas examined in our experiments we also found hyperthermic en-

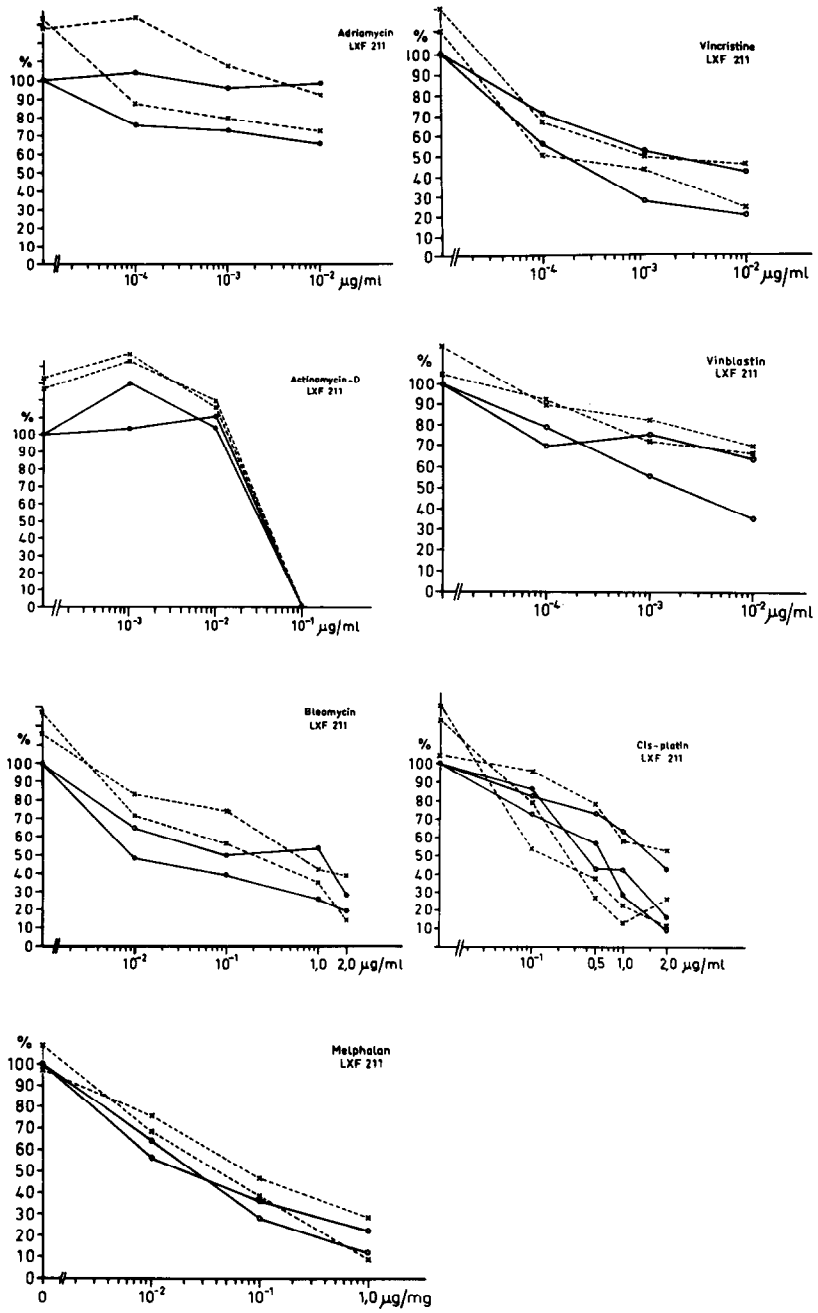

Fig. 5.

hancement of melphalan. The other tumors did not show a difference between normo- and hyperthermic incubation.

Cisplatin is described as a drug that is enhanced in a linear way with increasing temperature [21, 22]. Therefore an enhancement should be expected at 40.5°C. In contrast to these observations, in our tumor specimens we could not demonstrate a difference between normo- and hyperthermic incubation.

The vinca alkaloids are mitotic phase-specific acting drugs that bind to the microtubules of dividing cells. Hyperthermia has been shown to disrupt the tubular structures and disassemble the microtubules at 41°C [23]. A hyperthermic enhancement might therefore be expected. Indeed, we found hyperthermic enhancement in three tumors with vincristine, whereas no enhancement was seen with vinblastine. Using CHO-cells at 42.5°C, Herman [24] found an additive lethality with vinblastine. Mizuno *et al.* [15] found no thermal enhancement of vincristine at 42°C using a mouse leukemia and a mammary carcinoma cell line, but again, the different test systems used have to be considered. When testing drugs and hyperthermic effects *in vitro*, it is of

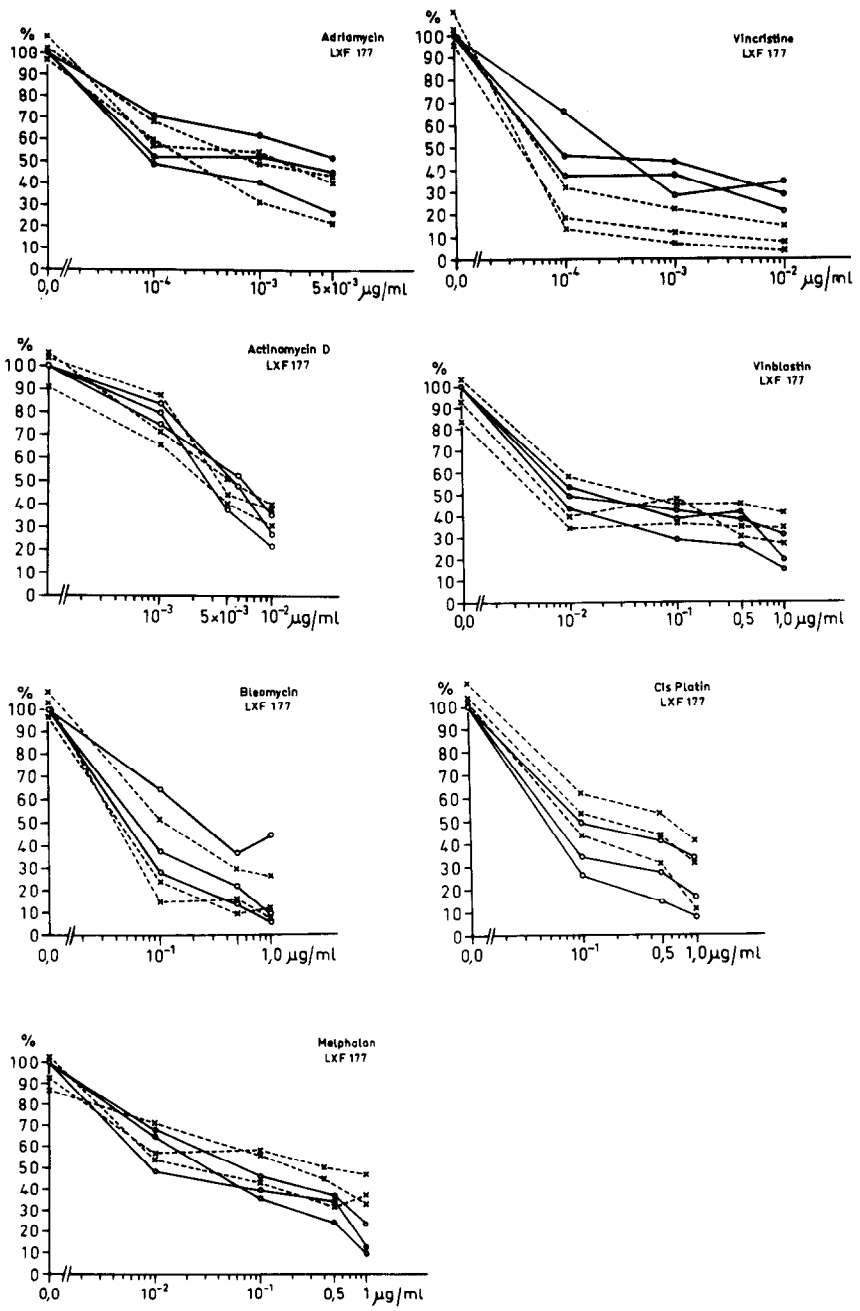

Fig. 6.

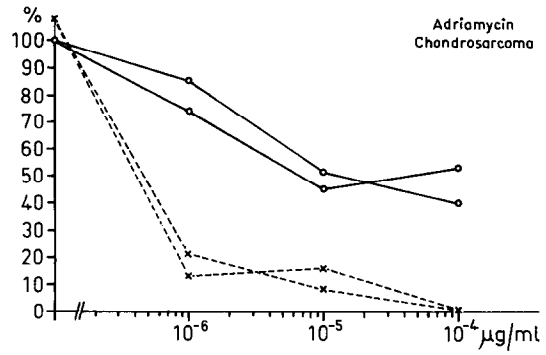

Fig. 7.

great importance that the drug effect is not altered by factors other than hyperthermia. pH values *in vitro* are especially important. Hahn and Shiu [25] could demonstrate that enhancement of cytotoxic drug effects is critically dependent upon the milieu. Under the employed conditions, i.e. a 2-hr exposure to 40.5°C, there was no difference between normo- and hyperthermic incubation.

With the use of a human tumor colony-forming assay, we could demonstrate that each tumor has an individual sensitivity pattern with respect to the drugs tested. An enhancement of drug effects can be obtained at 40.5°C. But this is quite obviously an individual property of each tumor. The pattern of drug effects and hyperthermic enhancement is reproducible individually, but cannot be transformed to all tumors of the same histologic type.

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