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Treatment of Human Clonogenic Tumor Cells and Bone Marrow Progenitor Cells with Bleomycin and Peplomycin Under 40.5°C Hyperthermia *In Vitro*

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Abstract—*Tumor cells derived from 13 different individual human tumors were plated in a colony forming monolayer assay. The effect of bleomycin and peplomycin on colony formation was assessed in normothermic conditions and after a hyperthermic treatment at 40.5°C for 2 h at the beginning of the culture. In three out of the 13 tumor samples (two colon carcinomas, one malignant melanoma), hyperthermic incubation resulted in a thermal enhancement of the effects of bleomycin and peplomycin.*

In addition, human bone marrow progenitor cells (CFU-C) were subjected to the same procedure. Peplomycin proved to be less toxic to CFU-C than bleomycin. In samples from eight different donors, homogeneous dose–response curves were observed. There was no difference between normo- and hyperthermic incubation.

INTRODUCTION

BLEOMYCIN is a mixture of glycopeptides differing only in their terminal amine groups [1–3] which is widely used in cancer therapy. Hahn *et al.* [4] were the first to demonstrate a thermal enhancement of the cytotoxic effect of bleomycin using experimental cell lines. They suggested that this effect resulted from an inhibition of the repair mechanism of the cell by hyperthermia.

Peplomycin is an analog that differs by a single terminal residue which is not present in bleomycin [3]. Both drugs act by causing DNA strand breaks [5]. It was the aim of this study to test the possibility of a thermal enhancement of bleomycin and peplomycin in several human tumors *in vitro*. Using human tumor samples, the individuality of each tumor concerning the sensitivity to cytotoxicity of drugs can be taken into account. In addition, the effect of bleomycin and peplomycin on human bone marrow progenitors (CFU-C) was tested under normothermic and hyperthermic conditions.

Hyperthermic effects in most cases are tested at temperatures above 42°C with respect to local hyperthermia. For the treatment of disseminated malignancies, whole body hyperthermia is of interest with the aim of enhancing the drug effects systemically. In this modality the temperature must be kept below 42°C. We therefore have tested a temperature which is achievable clinically without any risk for the patients under whole body hyperthermia conditions, i.e. 40.5°C for a period of 2 h.

MATERIALS AND METHODS

Tumor colony forming assay

Tumor cells were plated as described previously [6]. Briefly, a single cell suspension was obtained by mechanical disaggregation under sterile conditions. The viability of the cells was assessed by trypan blue exclusion. 1×10^5 viable cells were plated in 30% fetal calf serum (FCS, Boehringer Mannheim) in Iscove's modified Dulbecco's medium (IMDM Gibco, Karlsruhe). Methylcellulose at a final concentration of 0.9% (w/v) was used as a viscous support. Drugs were added at concentrations between 10^{-2} and 10.0 μg/ml. Incubation was performed at 7.5% CO_2 in a moist atmosphere for 8–10 days. Then colony formation was examined under an inverted microscope. The drug effect was expressed as the percentage reduction of colony formation as a function of drug concentration as

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Dedicated to Professor Dr G. W. Löhr on his 65th birthday.

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described by Salmon *et al.* [7]. Single cell suspensions from each tumor with methylcellulose, IMDM and FCS were mixed in two separate tubes. From each tube two dishes both for normo- and hyperthermic incubation were plated.

Cultivation of human bone marrow progenitors (CFU-C)

Bone marrow cells from healthy volunteers were aspirated from the iliac crest into heparinized syringes. Mononuclear cells of a density of less than 1.077 g/ml were separated by density centrifugation in Ficoll Hypaque (Farmacia, Upsala). Cells were washed and plated at a concentration of 2×10^5 cells/ml in 30% fetal calf serum, Iscove's modified Dulbecco's medium (Gibco) in methylcellulose of 0.9% (w/v). The final volume was 1 ml per culture dish. Cultivation was performed at 7.5% CO_2 in a moist atmosphere. Colony formation was evaluated after 10–12 days [8].

Tumors

Ten tumor probes were used immediately after surgical resection. The tumor samples had a size of 0.5–1 cm^3. The pieces were transported in cold Iscove's modified Dulbecco's medium from the Department of Surgery and processed immediately after arrival in the laboratory. Three samples were taken from nude mice (NMR I) (Laboratory Dr Fiebig, Freiburg) after the second and fourth passages. The animals were kept under laminar flow conditions and were fed with the mouse diet C 14 (Atrumin, Lage, F.R.G.). The animals were sacrified in the laboratory; the tumor samples were processed immediately afterwards. The size of the tumors was *ca* 1 cm in diameter.

Drugs

Bleomycin and peplomycin were supplied by courtesy of Dr P. Engel (Mack Illertissen, F.R.G.). The drugs were dissolved in distilled water. All experiments were performed using the same lots of bleomycin and peplomycin respectively. Drug exposure was performed continuously.

Hyperthermic treatment

In order to have hyperthermic conditions that are compatible with clinical applications, we applied a temperature of 40.5°C for 2 h. The hyperthermic culture dishes were placed in an incubator at 40.5°C for 2 h at the initiation of the cultures. Afterwards the dishes were placed in a 37°C incubator for the rest of the incubation. The temperature was monitored with an electronic thermosensor (ETW, Waldkirch) in a sham culture dish containing the same amounts of methylcellulose and medium as the culture dishes with cells.

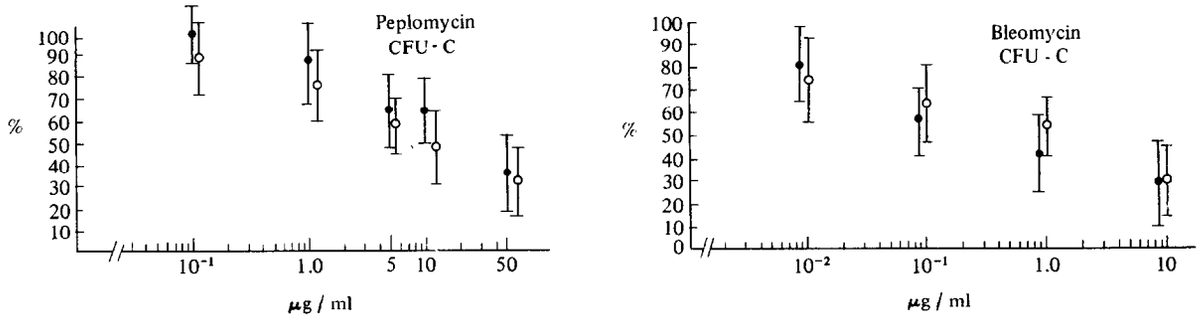
RESULTS

Bone marrow cells

Human granulocytic progenitor cells were exposed to bleomycin and peplomycin under normothermic and hyperthermic conditions at 40.5°C for 2 h. The plating efficiency was 82 ± 17.3/10^5 cells plated at 37°C and 77 ± 21/10^5 cells plated after a 2 h 40.5°C treatment. No thermal toxicity of hyperthermia could be seen. The dose–response curves for CFU-C with bleomycin and peplomycin under normo- and hyperthermic conditions are shown in Figs. 1 and 2. There was no difference between normothermic and hyperthermic treatment. The dose–response curves were established with marrow samples from eight different donors. All samples showed homogeneous dose–response curves. The high drug doses, necessary for a reduction of CFU-C growth, confirm the observation that bleomycin and peplomycin are not toxic to the bone marrow in clinical use [9–11]. A comparison of the two dose–response curves reveals that CFU-C formation is less suppressed by peplomycin than by bleomycin.

Tumor cells

Thirteen samples from different human tumors were plated in a methylcellulose monolayer under normothermic and hyperthermic conditions. The plating efficiency of the tumors under normothermic and hyperthermic incubation is shown in Table 1.



Figs. 1 *and* 2. *Reduction of colony formation for human bone marrow progenitors by bleomycin and peplomycin. The effect of the drugs is expressed as percentage reduction of colony formation as a function of drug concentration. The closed circles indicate the normothermic incubation, the open circles indicate the hyperthermic cultures* (40.5°C *for* 2 *h*). *The standard variation is indicated by the error bars* ($n = 8$).

Table 1. *Number of colonies/10^5 cells plated indicates the highest and the lowest number of colonies out of four control dishes without cytostatic drugs. The average of these four values was set as* 100%

	Patient	No. of colonies/10^5 cells plated		Thermal enchancement	
		Normothermic	Hyperthermic	Bleomycin	Peplomycin
Malignant melanoma	N.Z.	108/120	92/117	−	−
	B.St.*	179/206	168/192	+	+
	S.N.*	58/67	52/62	−	−
	G.P.*	110/118	94/102	−	−
Myosarcoma	M.F.*	60/65	66/72	−	−
	D.H.	62/67	57/63	−	−
	A.K.*	55/60	49/53	−	−
	F.D.	63/71	74/89	−	−
Colon carcinoma	R.Hu.*	156/169	162/171	+	+
	F.H.*	224/241	212/226	+	+
Squamous cell carcinoma of the lung	M.W.	91/116	98/105	−	−
	G.K.	94/110	92/105	−	−
Adenocarcinoma of the gall bladder	M.F.	117/130	124/132	−	−

* Indicates tumors in which peplomycin showed a greater cytotoxicity than bleomycin.

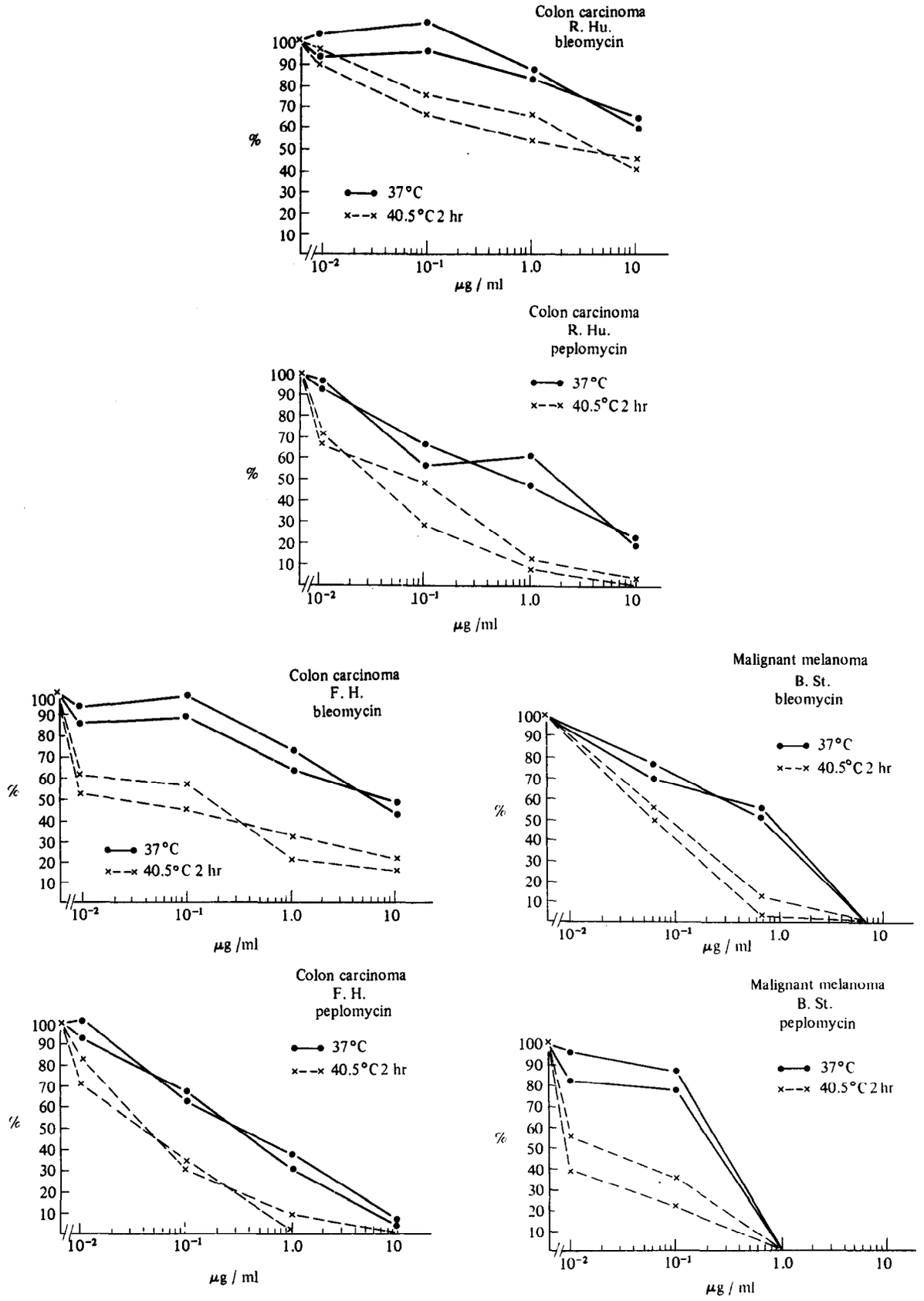
There is no significant difference between normothermic and hyperthermic exposure. In contrast to the homogeneous reaction of the normal bone marrow cells, the tumors showed individual heterogeneous drug sensitivity. In seven of the 13 samples, peplomycin proved to be more toxic than bleomycin. In three samples a thermal enhancement of both drugs was observed (Figs. 3–5). An example of a tumor showing no thermal enhancement is shown in Fig. 6. A thermal enhancement was observed when the difference between dose–response curves obtained under normothermic and hyperthermic conditions was greater than the variability among the dose–response curves observed under normothermic incubation or hyperthermia respectively. In the cases where no thermal enhancement could be observed, the dose response curves obtained under 37 and 40.5°C proved to be within the normal range of colony formation in this assay.

In one colon carcinoma (Fig. 3) (patient R.Hu.), colony formation under bleomycin at a concentration of 10 μg/ml was reduced to 58 and 63% compared to controls at 37°C whereas under hyperthermic incubation colony formation was reduced to 43 and 38% respectively. Using peplomycin, the reduction of colony formation was more pronounced. At a concentration of 10 μg/ml at 37°C a reduction to 20 and 25% of controls was observed, whereas under hyperthermia, colony formation was reduced to 3% and zero compared to control growth. In the other colon carcinoma (patient F.H., Fig. 4) under bleomycin colony formation was reduced to 48 and 41% whereas at 40.5°C colony formation was reduced to 19 and 13% of control. Again, peplomycin was more toxic than bleomycin. At 37°C at a concentration of 10 μg/ml colony formation was reduced to 8 and 5% respectively whereas after 40.5°C treatment at 10 μg/ml no colony formation was seen. At 1.0 μg/ml in one probe colony formation was reduced to 10 and 1% respectively. The thermal enhancement is more pronounced in the case B.St. (Fig. 5) with cells derived from a malignant melanoma. Using bleomycin, at 37°C, at 1.0 μg/ml colony formation was reduced to 53 and 49% of control growth whereas after 40.5°C treatment at this concentration the colony formation was reduced to 11 and 3% of control. Also in this tumor peplomycin proved to be more cytotoxic than bleomycin. At a drug concentration of 10^{-1} μg/ml a reduction to 88 and 78% of control was seen whereas at hyperthermic conditions colony formation was reduced to 36 and 21% respectively. At a concentration of 1.0 μg/ml colony formation was inhibited completely.

In one case with a carcinoma of the gall bladder, under bleomycin no difference between normothermic and hyperthermic incubation was seen. Under treatment with peplomycin, colony formation was reduced to 16 and 24% of control at 10 μg/ml whereas at 40.5°C the colony formation was less, reduced to only 50 and 58% of control growth. In this case a protective effect of hyperthermia might be considered (Fig. 7). The same observation was made in our laboratory with one malignant Schwannoma under 4-epiadriamycin and melphalan treatment (data not shown here).

DISCUSSION

The human tumor clonogenic assay was developed as a tool in order to test the individual drug sensitivity of human tumors to cytostatic agents. A number of problems have been described that make it difficult to use this assay in clinical routine work [12, 13]. For the investigation of



Figs. 3–5. Dose–response curves of human tumor colonies derived from individual human spontaneous tumors. The initials of the patients, the drug and the type of the tumors are indicated in the charts. The drug effect is expressed as percentage reduction of colony formation as a function of drug concentration. The closed circles and the solid lines indicate the normothermic incubation, the × and the dotted lines indicate the hyperthermic incubation (40.5°C, 2 h at the initiation of the cultures).

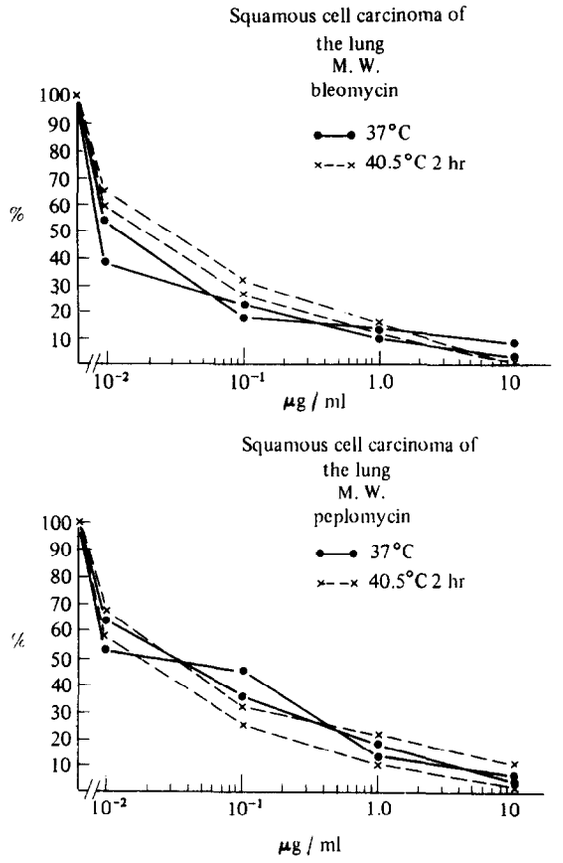


Fig. 6. Example for dose–response curves from a tumor treated with bleomycin and peplomycin showing no difference between normothermic and hyperthermic treatment. For explanation see legend to Figs. 3–5.

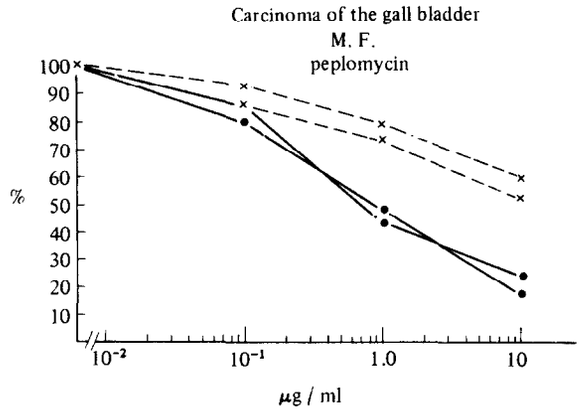


Fig. 7. Dose–response curves from a carcinoma of the gall bladder. At hyperthermic treatment with peplomycin (×--×) colony formation was less inhibited than at 37°C (●—●).

special problems, however, the human clonogenic assay proved to be useful, for example, in the preclinical level for testing new agents [14–16]. For the examination of thermochemosensitivity this assay was used by several groups [17–19]. In this study the purpose was to compare the cytotoxicity of bleomycin and peplomycin and the possibility of a thermal enhancement of their cytotoxic effect.

It is well established that the cytotoxic effect of bleomycin can be enhanced by hyperthermia [4, 20–22]. Most of these experiments were performed using cell lines. Hahn *et al.* [4] described a thermal enhancement of bleomycin occuring at a temperature level above 42.5°C but not below this level. In contrast to drugs that can be enhanced at lower temperatures in a linear way such as cisplatin and alkylating agents, bleomycin was classified as a 'threshold drug' with respect to hyperthermic enhancement [23]. With the use of human tumor material it could be demonstrated that the different tumors were heterogeneous in their drug sensitivity as well as in their sensitivity to thermochemotherapy. In contrast to the observations made, using experimental cell lines, in our system a thermal enhancement can be observed at the comparatively low hyperthermic level of 40.5°C. In some tumors peplomycin proved to be more toxic than bleomycin (see Table 1). In three cases ut of the 13 tumor samples tested, a thermal enhancement of bleomycin as well as of peplomycin was observed. It is noted that bleomycin causes double strand breaks whereas peplomycin usually causes single strand breaks [5]. The observation that in these three cases thermal enhancement occurs with both drugs suggests that the mechanism of thermal enhancement is similar in both drugs.

Previous experiments [9–11] demonstrated the low bone marrow toxicity of bleomycin. Comparing the dose–response curves for bleomycin and peplomycin, we could show that peplomycin is less toxic to CFU-C than bleomycin. No difference between normo- and hyperthermic incubation was observed. In contrast to the tumor samples that showed heterogeneous individual dose–response patterns, the dose–response curves for CFU-C showed homogeneous curves.

In conclusion, the effect of bleomycin and peplomycin can be enhanced by a comparatively short incubation time of 2 h and a moderate hyperthermia of 40.5°C. The possibility of enhancement seems to depend upon the individual properties of the tumor. Clinical studies will be necessary to elucidate if an *in vitro* test for hyperthermic enhancement will be useful for the prediction of thermochemosensitivity in the human situation.

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