J. steroid Biochem. Vol. 23, No. 6B, pp. 1163–1167, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

IV. Experimental Models and New Perspectives in a Combined Approach to Breast Cancer

CHEMOTHERAPY AND HORMONAL THERAPY OF MOUSE MAMMARY TUMORS

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Summary—Single Cisplatin (Platinol®) injections i.p. at 10 mg/kg dose level (LD_{10}) caused a marked growth inhibition of both hormone dependent and independent GR mouse mammary tumors (reduction in tumor volumes of 86 and 85%, respectively). However this treatment caused severe toxic side effects including loss of mouse body weight and deaths of some of the animals. When Cisplatin (pure) was administered s.c. in a single cholesterol pellet, no such toxic effects were observed even when Cisplatin levels up to 30 mg/kg were applied. The latter dose caused 29% reduction in tumor volume of hormone independent tumors. However this inhibitory effect was temporary, since regrowth of the hormone independent tumors started again 8 days after application of the single Cisplatin pellet. These results show that continuous Cisplatin administration in pellets s.c. is less toxic than injection i.p., but that the inhibition of mammary tumor growth is also less marked.

INTRODUCTION

Experimental mammary tumors in rodents are useful models for investigating the properties of hormone dependent malignant neoplasms. In 1974 we reported that hormone responsive (HR) mammary tumors of GR mice are heterogeneous populations of ER positive and ER negative tumor cells, and that only the ER positive cells respond to endocrine ablation [1]. The HD and HR tumors on average have higher ER, PgR and PLR contents than the HI tumors. AR levels are insignificant in GR mouse mammary tumors [2, 3]. In subsequent studies the following general conclusions were reached regarding this model system [4–6]:

(1) Hormone dependent GR mammary tumors progressively become less hormone dependent during serial transplantation due to the emergence of successively more malignant subpopulations of autonomous cancer cells. This selection initially is mainly due to a better transplantability of HI cells compared to HD cells, but in late transplant generations there is also the added effect that the more malignant HI subclones that then arise also have increased growth rates. The latter malignant cells often show sarcomatoid features.

(2) ER and PgR levels in HI tumors on average are significantly lower than those in HD or HR tumors, and therefore the absence of these hormone receptors is a good marker for hormone independence. However, there is no significant difference in hormone receptor levels between HD and HR tumors, indicating that ER or PgR measurements are not sufficiently sensitive to signal the presence of marked amounts of autonomous cells within HR tumors. The HR tumors of GR mice in a sense might therefore be comparable to ER false positive cases of breast cancer that are observed in the clinic, i.e. human tumors that are ER positive but that do not give objective remission after endocrine therapy. We have therefore proposed that the lack of objective response of many ER positive breast cancers is mainly due to the intrinsic heterogeneity of these tumors, and to the fact that small amounts of ER negative cells within the tumor mass that do not markedly lower ER assays, yet are sufficient in number to prevent objective remission after endocrine treatment [2].

In addition, as has been suggested by other authors some cells may have ER that can bind estrogen but then cannot bind to nuclear DNA; such cells show up as ER positive non-responding cells [7].

(3) In HD and HR mammary tumors of GR mice the levels of PgR are on average higher than those of ER. In the HI tumors the PgR level is practically zero, whereas in these tumors low but significant levels of ER are detectable. For this reason the level of PgR in mouse mammary cancer is a better prognostic marker than that of ER. The reason that in human breast cancer, PgR levels appear to be better prognostic markers than ER levels [8] may therefore not necessarily be due to presence of some cells that have ER that can bind estradiol but then cannot translocate, as has been proposed by Horwitz *et al.*[7], but merely due to the fact that PgR > ER in HD cells, and PgR < ER in HI cells [2].

(4) That HR mammary tumors of GR mice are mixtures of different cell clones was also demonstrated by examining mouse mammary tumor virus

Abbreviations: HD, hormone dependent; HR, hormone responsive; HI, hormone independent; ER, estrogen receptor; PgR, progesterone receptor; PLR, prolactin receptor; AR, androgen receptor.

(MMTV) proviral DNA in tumors of successive transplant generations. By using the MMTV-DNA as a genetic marker for cell clones we demonstrated that HD tumors can already contain significant amounts of autonomous cells, and that the levels of these cells increase during serial transplantation and the concomitant progression of the tumors [9].

(5) The heterogeneity of mammary cancer suggests that chemo-hormonal therapy might be more effective in inhibiting tumor growth than either treatment given singly [10]. Investigating this with GR mouse mammary tumors, we found that a combination of cyclophosphamide and tamoxifen was more effective in inhibiting growth of HD tumors than either treatment given alone [11]. Monohydroxytamoxifen, when given in pellets s.c. caused more growth inhibition of HD mammary tumors than tamoxifen. (Since 4-monohydroxytamoxifen is broken down more quickly than tamoxifen in the circulation when injected, we administered both drugs s.c. in order to compare their relative effect when administered continuously) [12].

(6) There is no significant difference between response to chemotherapy between the HD and HI mammary tumors of GR mice. Growth inhibition by this cytostatic drug varied greatly between tumors from different lines and was not related to ER content [6]. However, rapidly growing tumors appeared to be slightly more sensitive to cyclophosphamide than slowly growing tumors [13]. Cyclophosphamide treatment appeared to cause most growth inhibition of GR mammary tumors at their transition between hormone dependence and independence. This might be due to the fact that at this stage the autonomous cells within the tumor start to increase rapidly, and these cells therefore might be more sensitive to the cytotoxic drug at this point [14].

Recent clinical trials indicate that by giving certain groups of patients with advanced breast cancer combined chemo-hormonal therapy instead of the single treatments, improvement in response rates and duration of tumor-free interval can be achieved. However, as yet there is no indication that the combined treatment also caused an increase in ultimate survival of patients. This could mean that the cytotoxic drugs that we are using are not destroying the most malignant autonomous cell clones. Future studies should therefore be directed at identifying these potentially most dangerous cells, and of finding new drugs to destroy them. An alternative study would be to investigate whether drugs which have been shown to be effective in the treatment of other cancer types could also be used to combat breast cancer. Cisplatin has demonstrated a high order of activity in carcinomas of the testis, bladder, cervix, ovary, head and neck. However, although it has also shown some activity against breast cancer, this is only at dose levels that cause toxicities of nausea, vomiting and inanition [15]. The following experiments were carried out to determine the effect of cisplatin on the growth of GR mouse mammary tumors.

EXPERIMENTAL

Animals and tumors

Mammary tumors were induced in GR mice and serially transplanted in (O20 × GR)F_1 hybrid mice as described previously [1].

Chemicals

The Cisplatin used for injection was Platinol® from Bristol–Myers. Diluted in water (10 ml) this contains 1 mg Cisplatin (*Cis*-diammineddichloroplatinum), 9 mg NaCl, 10 mg mannitol (pH 2–3). Adriamycin was obtained from Pharmitalia, and PALA (*N*-phosphonacetyl-L-aspartate) from the National Cancer Institute, Bethesda (MD).

RESULTS

Cisplatin treatment of mouse mammary tumors

We have carried out studies to determine whether the toxic side effects of Cisplatin could be diminished by altering the method of drug application. Figure 1 and Table 1 show the results of a study in which the effect of Cisplatin on GR mouse mammary tumors was compared with that of Adriamycin and PALA (*N*-phosphonacetyl-L-aspartate).

Hormone dependent GR mammary tumors were grafted into 7 groups of 10 castrated (O20 × GR)F_1 mice which were treated continuously with estrone plus progesterone [1]. On day 20 after grafting, the

Table 1. Effect of Cisplatin, adramycin and PALA on the growth of hormone dependent GR mouse mammary tumor grafts, and on mouse body weights

Treatment	Dose (mg/kg)	Tumor weight (g) on day 26	Body weights (g)* B_1	Body weights (g)* B_2	No. of deaths
Untreated	—	3.47 ± 0.89	31.15 ± 4.70	33.02 ± 5.05	1/10
Cisplatin†	10	0.49 ± 0.17	29.95 ± 3.65	28.57 ± 2.69	2/10
	5	1.77 ± 0.44	29.59 ± 4.05	33.73 ± 2.79	0/10
Adramycin	12.5	1.38 ± 0.42	30.64 ± 5.48	32.40 ± 3.67	0/10
	6.25	2.09 ± 0.54	29.68 ± 2.41	32.73 ± 2.45	1/10
PALA	280	3.03 ± 0.60	30.00 ± 3.40	32.91 ± 3.86	0/10
	140	3.48 ± 0.61	30.82 ± 3.77	32.89 ± 2.90	0/10

*B_1, body weight on day tumor was grafted; B_2, body weight (including tumor) on day the mice were killed (day 26).
†Platinol®.

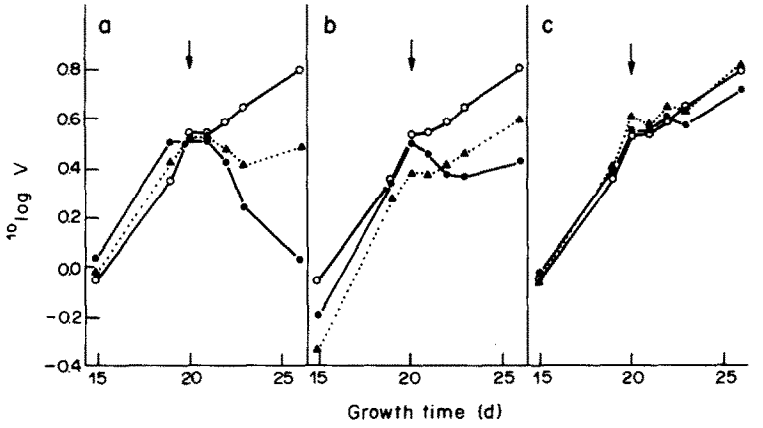

Fig. 1. Effects of (a) Cisplatin; (b) Adriamycin; (c) PALA on the growth of hormone dependent GR mouse mammary tumor transplants. Treatment was given by a single injection i.p. of the drug on day 20 after grafting of the tumor tissue (arrow). Treatment with LD_{10} dose (●——●) or 1/2 LD_{10} dose (▲ . . . ▲). Untreated controls (○——○); the same controls were used for all treatment modality groups. ^{10}Log V is plotted on the ordinate, where V is tumor volume (cm^3).

treatment groups received single LD_{10} or 1/2 LD_{10} dose of cytostatic agent i.p., while control mice were not treated. (LD_{10} values for Cisplatin, Adriamycin and PALA are 10, 12.5 and 280 mg/kg, respectively.) Tumor dimensions were measured with a vernier caliper. The mice were killed on day 26, and the tumor outgrowths excised and weighed (Fig. 1 and Table 1).

Mice that were treated with a single dose of 10 mg Cisplatin/kg on day 20 had significantly lower tumor volumes than untreated controls on days 22 ($P = 0.005$), 23 and 26 ($P < 0.005$), while tumor weights on day 26 were reduced by 86% compared to controls ($P < 0.005$). Mice treated with 5 mg Cisplatin/kg had smaller tumor volumes than controls on day 22 ($P = 0.01$), 23 and 26 ($P < 0.005$) and gave lower tumor weights than controls ($P = 0.005$) on day 26.

Results with Adriamycin were less marked than those with Cisplatin. Nevertheless, mice treated with 12.5 mg Adriamycin/kg had significantly lower tumor volumes than untreated controls on days 22, 23 and 26, and gave lower tumor weight yields ($P < 0.005$). Those treated with 6.25 mg Adriamycin/kg had lower volumes than those of controls on days 20 ($P = 0.025$), 21 ($P = 0.008$), 22 ($P < 0.005$), 23 ($P = 0.007$) and 26 ($P < 0.005$), while tumor weights were also lower ($P < 0.005$). Treatment of mice with PALA at 280 or 140 mg/kg dosage did not significantly affect mouse mammary tumor growth.

In order to evaluate the toxicity of the cytostatic agents, we measured their effect on the body weights of the mice. Table 1 shows that the weights of untreated mice increased from day 0 to day 20. Treatment with the LD_{10} dose of Cisplatin on day 20 caused a drop in body weight and the death of 2 mice out of 10. In the other treatment groups, the chemotherapy did not cause a decrease in body weights. These data therefore show that an LD_{10} dose of Cisplatin causes a very significant growth inhibition of hormone dependent GR mouse mammary tumors, but that the loss in body weight of the treated mice, and the death of 2/10 mice indicates a marked toxicity of Cisplatin at this dosage level.

Administration of Cisplatin in pellets s.c.

The following experiments were carried out to determine whether Cisplatin, administered in pellets s.c. might be less toxic than that given i.p., but still inhibit mouse mammary tumor growth. Five groups (Groups A–E) of castrated (O20 × GR)F_1 mice (10 mice per group) received hormone independent GR mammary tumor grafts, and on day 10 of tumor growth were treated with Cisplatin. Group A mice received an LD_{10} dose (10 mg/kg body weight) of Cisplatin (Platinol®, Bristol). Groups B, C and D received cholesterol pellets s.c. containing 3.3 mg, 10 mg and 30 mg portions of Cisplatin (pure, Bristol) per kg mouse body weight, respectively. Mice of group E received cholesterol pellets s.c. to which no Cisplatin had been added (controls).

Figure 2a and Table 2 show that i.p. injection of 10 mg Cisplatin/kg caused a sharp decrease in tumor volume by 85% on day 8 after the injection compared to untreated controls. However death of $\frac{5}{10}$ of the mice occurred on day 4 after the i.p. injection of Cisplatin; body weights of the remaining mice dropped by 6 g by day 18 of tumor growth; beyond that date body weights of the mice increased slightly but remained significantly below those of controls (group E).

Administration of Cisplatin in cholesterol pellets inserted s.c. did not inhibit mouse mammary tumor growth to the same extent as i.p. injection when calculated per mg Cisplatin administered per kg mouse body weight, but gave less general toxicity. None of the mice treated with Cisplatin in pellets s.c. (groups B, C and D) died due to this treatment. Dosages of 3.3 or 10 mg Cisplatin per kg body weight

Table 2. Effect of a single injection of Cisplatin and of continuous Cisplatin administration by pellets s.c. on the growth of hormone independent GR mouse mammary tumors

Treatment group*	Tumor yield		Body weight (g)‡		
	Day	Weight (g)†	B_1	B_2	No. of deaths
A	27	2.95 ± 1.02	28.70 ± 2.39	25.16 ± 0.79	5/10
B	18	4.32 ± 0.47	28.82 ± 2.00	30.70 ± 2.28	0/10
C	18	3.97 ± 1.04	27.96 ± 2.27	29.09 ± 1.80	0/10
D	22	4.11 ± 0.65	28.26 ± 3.03	28.93 ± 2.66	0/10
E	19	4.72 ± 1.13	27.50 ± 2.01	29.20 ± 1.98	0/9

*Group A, 10 mg Cisplatin (Platinol®) per kg by injections i.p.; Groups B, C, D, cholesterol pellets s.c. containing 3.3 mg, 10 mg, and 30 mg Cisplatin (pure) per kg, respectively. Group E, cholesterol pellets s.c. without Cisplatin (controls).

†Average weights ± SD.

‡B_1, body weight on the day the tumor was grafted; B_2, body weight (including tumor) on the day the mice were killed. Average weights ± SD.

in pellets s.c. did not inhibit tumor growth. However a dose of 30 mg/kg in pellets inserted s.c. (group D) gave a significant inhibition of tumor growth (38% reduction in tumor volume on day 18 compared to controls). The tumor volumes of group D mice differed significantly from those of the untreated mice (group E) on days 14 ($P = 0.001$), day 17 ($P = 0.001$) and day 18 ($P = 0.006$). Of interest was the finding that the decrease in body weights minus tumor burden of group D mice paralleled that of untreated controls (Fig. 2b). This suggested that the decrease in body weights of the mice that were given 30 mg Cisplatin/kg in pellets s.c. was not due to the Cisplatin treatment but to the tumor burden.

The latter finding prompted us to investigate the effect of mammary tumor burden on mouse body weight. For this study we used the data obtained with the control groups (untreated mice) of the experiments described above. The body weight of each mouse was noted at the beginning of the experiment (B_1), its weight (including tumor) at the end of the experiment (B_2), and the weight of the tumor that was excised at the end of the experiment (T_2). Figure 3 shows the plot of T_2 versus $(B_2 - T_2 - B_1)$. The data from 20 mice (11 with HD and 9 with HI tumors) indicated that an increase in tumor burden of 1 g resulted in a 2.4 g decrease in the weight of the

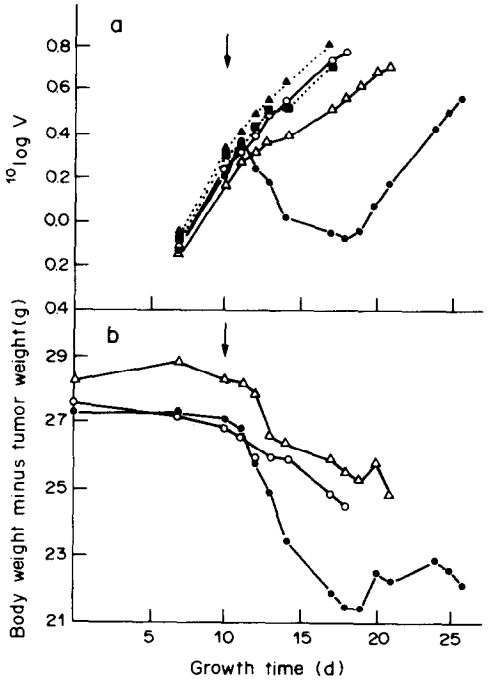

Fig. 2. Effect of Cisplatin on the growth of hormone independent GR mouse mammary tumor transplants (a), and on body weights of the tumor-bearing mice (b). Treatment with Cisplatin was given on day 10 after grafting the tumors in castrated (O20 × GR)F_1 mice (arrow). Treatment by i.p. injection of 10 mg Cisplatin/kg, ●——●. Treatment with cholesterol pellets s.c. containing 3.3 mg (▲ . . ▲), 10 mg (● . . ●) or 30 mg (△ . . △) Cisplatin/kg. Untreated controls, ○——○. V = tumor volume.

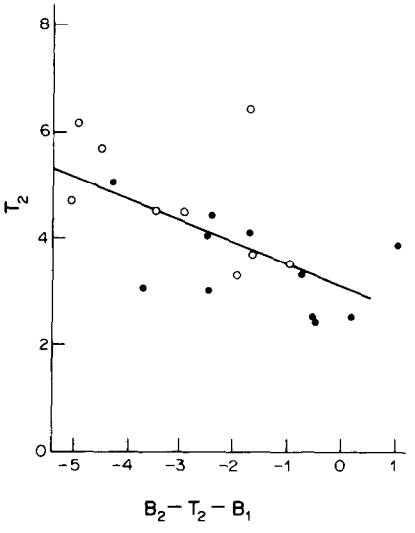

Fig. 3. Effect of tumor burden on the remainder of mouse body weight. Castrated (O20 × GR)F_1 mice were weighed (B_1), and hormone dependent or independent mammary tumor tissue was grafted [1]. When the outgrowths had reached an appreciable size, the mice were again weighed (B_2), and then killed and the tumor excised and weighed (T_2). All weights are expressed in grams. Hormone dependent tumors with growth time 26 days, ●——●; hormone independent tumors with growth time 18 days, ○——○. The straight line was drawn according to least squares ($P < 0.01$).

remainder of the mouse body, probably due to recruitment of nutrients by the tumor tissue, and toxic side effects by the tumor on mouse bodily functions.

DISCUSSION

The data presented here show that Cisplatin injected i.p. or administered in pellets causes inhibition of mouse mammary tumor growth. Some inhibition was also obtained with Adriamycin, but PALA had no effect. With Cisplatin pellets s.c. the dosage required to give a certain inhibition of tumor growth was higher than with Cisplatin injections i.p., but the toxicity of the pellets s.c. was relatively much lower. Thus up to 30 mg Cisplatin/kg could be given to mice in pellets s.c. without lethal effect or loss of body weight, whereas only 5 mg/kg could be given i.p. without these adverse effects.

A single dose of 10 mg Cisplatin/kg caused a marked growth inhibition of both hormone dependent and independent mammary tumors. This treatment caused about the same decrease in HD tumor volume as in HI tumor volume (86 and 85%, respectively) although the HI tumors used were more highly malignant than the HD tumors (untreated grafts of the HI tumors yielded outgrowths of 4.7 g in 18 days, whereas those from the HD tumors yielded outgrowths of 3.5 g in 26 days).

Figure 2 shows that 8 days after the administration of 30 mg Cisplatin per kg in pellets s.c. to hormone independent tumors, a 29% reduction was obtained compared to untreated controls. This treatment did not affect mouse body weights (Table 2).

In conclusion therefore our results show that continuous Cisplatin administration by cholesterol pellets s.c. is less toxic than single Cisplatin injections i.p., but that the inhibition of mouse mammary tumor growth is also less marked.

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