Effects of Intermittent Diethylstilbestrol Diphosphate Administration on the R3327 Rat Prostatic Carcinoma

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ABSTRACT
Clinical trials have utilized intermittent diethylstilbestrol diphosphate (DES) therapy in advanced symptomatic prostatic carcinoma to diminish the morbidity of standard endocrine therapy. To determine the effect of intermittent DES administration on the Dunning R3327 rat prostatic adenocarcinoma model, 60 days following tumor implant, 6 groups were randomly assigned: control (N = 8), castrate (N = 10), high dose DES (N = 8, 1.6 µg/ml DES continuously in drinking water), low dose DES (N = 10, 0.4 µg/ml continuously in drinking water), intermittent high dose DES (N = 10, 1.6 µg/ml DES in drinking water for 1 week, then off for 3 weeks), and intermittent low dose DES (N = 10, 0.4 µg/ml DES for 1 week, then off for 3 weeks). Results indicate that low or high dose DES, and intermittent low or intermittent high dose DES during the week of administration were able to reduce serum testosterone to castrate levels (0.1 ng/ml). After withdrawal of intermittent DES, serum testosterone returned toward control levels (1.0 ng/ml). Initial mean tumor burden between control and treatment groups was not significantly different. All DES exposed rats had a tumor volume at death (range, 15.6-18.3 cm³) smaller than control (mean, 25.4 cm³) or castrate (mean, 40.8 cm³) rats. Despite this, significant survival advantage from the time of randomization was achieved only in castrate (median survival, 331 days) or high dose DES (median survival, 359 days) groups compared to control (median survival, 225 days). Similarly, significant prolongation in tumor doubling time was achieved only by rats receiving castration or high dose DES. Intermittent DES administration controls tumor volume but does not provide a survival advantage. In this respect, intermittent DES is inferior to castration.

INTRODUCTION
Since 1941, endocrine therapy (1) has been the most effective treatment for patients with metastatic prostatic adenocarcinoma. Approximately 70-80% of patients will achieve symptomatic relief of bone pain and bladder outlet obstruction, as well as improvement in constitutional symptoms (2). Despite alleviation of symptoms, the Veterans Administration Cooperative Urological Research Group’s study of cancer of the prostate (3) and others (4) could not demonstrate a survival advantage for patients receiving early versus delayed endocrine therapy. Psychological disadvantages of bilateral orchectomy, and the physiological side effects of estrogen therapy, including cardiovascular toxicity, thromboembolic phenomena, gynecomastia, fluid retention, and loss of libido, introduce morbidity in standard methods of endocrine control (5). Moreover, questions exist concerning the optimal timing of endocrine therapy, the biological explanation for relapse following such treatment, and the relation of initial serum testosterone levels to duration of response to endocrine therapy.

Klotz et al. (6) demonstrated that patients responding to DES may achieve a durable symptomatic remission after the withdrawal of DES and a return of the serum testosterone to the normal range. In patients subsequently relapsing, remission could be reinduced with the resumption of DES.

The rationale for this study was to determine if an experimental basis for intermittent DES therapy could be demonstrated in the R3327 rat prostatic carcinoma. This tumor is slow growing, histologically well differentiated, and androgen sensitive. It differs from human prostate cancer in that it is seldom metastatic (7, 8). In this study, we compare the effects on tumor doubling time, tumor volume at death, and survival in a rat prostatic adenocarcinoma model utilizing intermittent and continuous DES administration at low and high doses, and castration.

MATERIALS AND METHODS
Fifty-six Fischer × Copenhagen F1 rats weighing approximately 250 g were obtained through the courtesy of Dr. N. Altman of the Papanicolaou Cancer Institute, Miami, FL. Prior to shipment to Memorial Sloan-Kettering Cancer Center, the rats received needle trocar implants of the R3327 tumor, of 2 mm³ in each flank. The rats were maintained in an animal facility with 12-h light, 12-h dark cycles, and rat chow and water ad libitum. Sixty days after receipt of the animals, all groups had tumors with a mean tumor range varying between 0.05 and 0.38 cm³, and weights ranging between 358 and 378 g. The rats were randomly assigned to 6 groups: control (N = 8), castrate (N = 10) (transcrotal castration was performed under i.p. pentobarbital anesthesia); water soluble DES1 H-DES (N = 8, 1.6 µg/ml DES continuously in the drinking water); and IL-DES (N = 10, 0.4 µg/ml DES in the drinking water for 1 week, off for 3 weeks); L-DES (N = 10, 0.4 µg/ml DES continuously in the drinking water); and IL-DES (N = 10, 0.4 µg/ml in the drinking water for 1 week, off for 3 weeks). In prior experiments, DES doses of 0.4 and 1.6 µg/ml had been demonstrated to reliably suppress serum testosterone when administered p.o. (9). Where indicated, transcrotal castration was performed under i.p. pentobarbital anesthesia.

Each separate flank tumor was measured weekly with microcalipers and tumor volumes were calculated by the formula \( V = \frac{1}{6} \pi \times w \times h \times 0.523 \). Tumor burden was defined as the sum of both flank tumor volumes per rat. Rats were weighed weekly and fluid intake was monitored. Carcass weight was calculated by subtracting the tumor volume (estimating tumor weight as 1 cm³ = 1 g) from the rat weight. Because DES can promote weight loss in rats (10), final tumor volume at death was calculated per 100 g body weight. Tumor doubling times were calculated by using the following formulas:

\[ \ln V_t = \ln V_i + kT \]

Where \( T \) is time in days. The doubling time was determined from the equation:

\[ DT = \ln \frac{2}{k} \]

with the slope \( k \) determined by linear regression analysis, where \( \ln V \) is the natural log tumor volume; \( V \) is the natural log initial tumor volume; \( k \) is the exponential growth constant; and \( DT \) is the tumor doubling line. These calculations were performed when individual tumors ranged from 1.6 cm³ up to 20.1 cm³, a period of exponential growth (11).

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2 The abbreviations used are: DES, diethylstilbestrol diphosphate; R3327 androgen dependent, well differentiated slow growing Copenhagen rat prostatic tumor originally described by Dunning; H-DES, high dose DES; L-DES, low dose DES; IL-DES, intermittent low dose DES; HH-DES, intermittent high dose DES.
Postmortem examination was performed in search of metastatic disease.

Retroorbital venipuncture was performed to obtain serum. Utilizing the protocol described by the manufacturer (Radioassay Systems Laboratories, Carson, CA), 125I radioimmunoassay determination of serum testosterone at 1 and 4 weeks was performed. The lowest detectable level of testosterone in this assay is 0.1 ng/ml.

The experiment has been followed for 614 days since the time of randomization into treatment groups, and 674 days since the time of tumor implant. Median survival was calculated and significance of differences in median survivals between groups was tested by the Wilcoxon rank sum test. Mean tumor volumes between groups were compared and differences were tested by using the Student's t test. Calculations and data storage were facilitated by the Memorial Sloan-Kettering Cancer Center Clin Computer system (New York, NY).

RESULTS

Serum testosterone was determined after 1 week of intermittent DES administration and 3 weeks after the withdrawal of intermittent DES. Rats receiving IH-DES and IL-DES had castrate levels of serum testosterone (testosterone less than 0.1 ng/ml) after 1 week of administration. Three weeks after the withdrawal of DES, the mean serum testosterone values in the IH-DES group [0.72 ± 0.16 (SE) ng/ml] and the IL-DES group [1.09 ± 0.31 ng/ml] approached those of control rats (1.01 ± 0.17 ng/ml). Rats receiving L-DES and H-DES and rats undergoing castration all had serum testosterone levels of less than 0.1 ng/ml.

Mean carcass weight as a function of time is illustrated in Fig. 1. During the initial 37 days of the experiment, rats in the L-DES and H-DES groups showed markedly decreased in mean carcass weights of 80 and 100 g, respectively, whereas rats in the other groups showed relatively less deviation from controls during this initial period.

The calculated doses of DES delivered to the rats were based on average daily fluid intake and are as follows: L-DES, 6 μg/day or 0.02 μg/g body weight/day; H-DES, 19.2 μg/day or 0.07 μg/g body weight/day; IL-DES, 6.4 μg/day or 0.02 μg/g/day; and IH-DES, 26 μg/day or 0.07 μg/g/day (6 μg/day is approximately equal to 1 mg/day in a 70-kg man).

Mean tumor volumes, tumor doubling times, and median survival data are shown in Table 1. There were no significant differences between the initial mean tumor burdens of the control rats and the other 5 groups. However, the initial mean tumor burdens in the IL-DES and IH-DES groups were significantly less than in the castrates at the start of the experiment (P < 0.05). The final tumor volume/100 g body weights at the time of death was greater in the castrate rats (40.84 ± 4.0 cm³) than all other groups (P < 0.05). Control rats final tumor volume/100 g body weight was significantly greater than L-DES, H-DES, and IL-DES (P < 0.05), and approached significance in the IH-DES group (P = 0.100). Tumor doubling time was significantly prolonged relative to control (31.9 ± 2.1 days) in castrate (54.0 ± 6.1 days) and H-DES (54.8 ± 3.6 days) (P < 0.005) treatment groups.

Survival status is represented in Fig. 2. A significant improvement in survival was achieved only in castrate (median survival, 331 days; P < 0.0001) and H-DES rats (median survival, 359 days; P = 0.0043) when compared to control rats (median survival, 225 days).

Postmortem examination revealed no evidence of visible metastatic disease in any group.

DISCUSSION

In this study, after 1 week of low or high dose DES administration to rats bearing the R3327 rat prostatic tumor, we were able to achieve a castrate level of serum testosterone, confirming the findings of Grossman et al. (9). IL-DES, IH-DES, and L-DES, while they did not cause a deleterious effect on survival when compared to control, were significantly less effective than castration or H-DES in terms of prolonging survival and tumor doubling time.

Data on mean carcass weight over time suggest that continuous H-DES or L-DES induces a catabolic state independent of the tumor effect most evident during the initial 37 days of the experiment. A catabolic estrogen effect was reported by Dunning et al. (10) who administered supraphysiological doses of DES in hopes of inducing malignancy. Tumor bearing rats from the other groups maintained weights comparable to control during this same time period. The total dose of DES delivered was similar in the IH-DES and L-DES, yet between these two groups, only L-DES caused a rapid decrease in weight.

Despite a considerable range in initial mean tumor burden, the control initial mean tumor burden did not differ significantly from that of the other groups. This difference may be a function of the trocar tumor implantation technique (11) or of differences in host-tumor interactions, but did not impact upon the segregation of final tumor volumes into groups based upon exposure to DES.

Data concerning the final tumor volumes per 100 g body weight at death suggests a cytotoxic effect of DES when administered either continuously or intermittently. Rats in the castrate group achieved significant improvement in survival over controls while bearing the largest mean final tumor volume at death. When compared to control, all DES treated groups had a significantly smaller final mean tumor volume, with the exception of the IH-DES group, which approached significance (P = 0.10).

In this study, no correlation between final tumor volume and survival was observed, suggesting that such was incidental to, rather than the cause of death. Tumors growing in a vital area, such as the liver or brain, as opposed to the flank, might have impacted more directly upon survival. In the latter case, DES delivered either intermittently or continuously might have been proven more favorable to survival than castration. The R3327 is unlike human prostatic carcinoma in its lack of metastatic potential, as was confirmed in our study. The exact cause of death in this model tumor system is not known, since metastatic spread does not occur (12). Speculation concerning the cause of death could involve many host-tumor interactions, yet it is
clear that castrated rats were better able to tolerate larger tumor burdens than rats receiving any form of DES.

Evidence for a cytotoxic action of estrogen in the Dunning R3327 tumor system has been reported. Rao incubated R3327-AT (anaplastic, hormone insensitive) and R3327 (hormone sensitive) cells in vitro with DES and 17β-estradiol and observed more than a 90% decrease in colonies in a clonogenic assay (13). In vivo, Lazan et al. (14) observed a dose dependent DES effect in lowering the number of metastatic colonies in the lungs of the R3327MAT-Lu (metastatic, anaplastic, hormone insensitive) tumor. Block et al. (15) also noted a decrease in tumor volume in the R3327G (poorly differentiated, hormone sensitive) tumor with DES exposure. The exact cytotoxic impact of the R3327 tumor for 60 days prior to manipulation with either castration or DES administration was not simply a function of testosterone withdrawal in inducing tumor growth retardation or improving survival. Ellis and Isaacs (16) demonstrated with castrated tumor bearing rats treated with small testosterone implants (producing a serum testosterone level of 0.2 ng/ml) that survival and tumor growth rate were not significantly different from castration (serum testosterone, 0.1 ng/ml), despite the fact that implanted rats had a serum testosterone twice as high as castrate rats. Trachtenberg (17) demonstrated that inhibition of tumor volume over a 9-week period could still be achieved when the serum testosterone was 8 times that of the serum testosterone level in castrates. It can be argued that during part of the time off DES, rats in the intermittent DES groups were still androgen deficient pending recovery of their pituitary-gonadal axis. By 3 weeks off DES, complete recovery was suggested by a return of the serum testosterone to normal.

Isaacs (11) demonstrated that maximum survival was achieved in the R3327H tumor bearing rats if castration occurred on the day of inoculation of tumor cells in a single cell suspension. Survival advantage progressively decreased with delay in castration such that if castration was performed 250 days following tumor implant, average survival was no different from intact tumor bearing controls. In our study, using rats that received tumor implants rather than single cells, and despite the 60-day delay in endocrine manipulation, improved median survival over control occurred only in the H-DES rats (120 days) and castrate rats (106 days). The initiation of intermittent DES administration (60 days after tumor implant) or the duration of its administration (1 week on, 3 weeks off), or some combination of both, may have contributed to the lack of improved survival in rats so treated.

In conclusion, utilizing the R3327 tumor implanted 60 days prior to the initiation of endocrine manipulation, tumor volumes at death in all DES exposed groups were less than those of control. Tumor control, but not better survival, was accomplished with intermittent DES. Survival did not correlate with final tumor volumes. Continuous suppression of serum testosterone may not be necessary for tumor volume control. Castrate and H-DES were superior to L-DES and intermittent DES in terms of survival in this model system.

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