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Protection by Testosterone from Fluorouracil-induced Toxicity without Loss of Anticancer Activity against Autochthonous Murine Breast Tumors[1]

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ABSTRACT

The present paper describes the specific amelioration of 5-flourouracil (FUra)-induced host toxicity (manifest in body weight loss, leukopenia, and mortality) by testosterone in the spontaneous, autochthonous $CD8F_1$ (BALB/c × DBA/$8F_1$) murine breast tumor model. Administration of testosterone did not affect the growth rate of these hormone-independent tumors, and, most importantly the antitumor activity of FUra was not reduced in testosterone-treated mice. Therefore, the net result of treatment with the combination of FUra and testosterone was an increase in the selective antitumor specificity of FUra.

INTRODUCTION

The efficacy of cancer chemotherapy is limited by the small differential in cytotoxic susceptibility between normal and neoplastic tissue. Because of this poor selectivity, it has been necessary to administer chemotherapeutic agents in suboptimal doses in order to avoid serious manifestations of toxicity in the host. More effective chemotherapy might be achieved if toxic manifestations in normal host tissues could be ameliorated selectively without compromising the anticancer activity of the drug. In such circumstances, it would be possible to utilize increased dosages and/or more aggressive treatment schedules necessary for eradication of the neoplastic cell population.

The present paper describes the specific amelioration of FUra[3]-induced host toxicity (manifest in body weight loss, leukopenia, and mortality) by testosterone in the spontaneous, autochthonous $CD8F_1$ murine breast tumor model. Testosterone initially was selected for this purpose on the basis of its reported ability to protect against the toxicity of another pyrimidine antagonist, deazauridine (2). Recently, testosterone has been shown to protect against toxicity associated with still another pyrimidine antagonist, (αS, 5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (17). We have previously reported that the administration of testosterone can protect against host toxicity without interfering with the anticancer activity of FUra-containing chemotherapeutic drug combinations in a primary syngeneic $CD8F_1$ breast tumor system (13, 14).

In the present studies, the amelioration of host toxicity by testosterone was evident when FUra was administered either alone or together with dThd. Significantly, the antitumor activity of FUra was not affected by testosterone, and therefore, the combination of testosterone and FUra resulted in an increase in the operational antitumor specificity of FUra.

MATERIALS AND METHODS

Murine Tumor System (Spontaneous, Autochthonous Mammary Carcinomas). For each therapy experiment, $CD8F_1$ hybrid mice bearing single spontaneous, autochthonous breast tumors arising during the preceding week were selected from our colony which has been described previously (12, 23). All tumors were measured, and the mice then were distributed among experimental groups so that mice carrying tumors of approximately equal weight were represented in each group. Tumor-free 3- to 5-month-old female $CD8F_1$ mice were used for toxicity studies.

Toxicity Measurements. Animal body weights were monitored immediately before and biweekly after the initiation of treatment. In experiments with tumor-bearing mice, the calculated tumor weight (see below), determined on each day that the mice were weighed, was subtracted from the total mouse weight to determine the actual body weight.

WBC levels in tail vein blood were determined either by standard visual counting procedure or electronically with a Fischer cell counter.

Chemotherapy. FUra was obtained from Hoffmann-La Roche Inc., Nutley, N. J., and dThd was obtained from Sigma Chemical Co., St. Louis, Mo. Each agent was dissolved in 0.85% NaCl solution immediately before use and was administered i.p. so that the desired dose was contained in 0.1 ml/10 g of mouse weight. However, when FUra was administered in doses above 100 mg/kg, the desired concentration was contained in 0.2 ml/10 g of mouse body weight. The agents were used in combination because the biological activity of FUra is potentiated by dThd (13–15, 21).

Testosterone. Testosterone enanthate (Delatestryl; E. R. Squibb & Sons, Princeton, N. J.) was administered in the thigh muscle in a volume of 0.05 ml.

Tumor Measurements. Two axes of the tumor (the longest axis, *L*, and the shortest axis, *W*) were measured with the aid of a vernier caliper. Tumor weight was estimated according to the formula

$$\text{tumor weight (mg)} = L\ (\text{mm}) \times [W\ (\text{mm})^2]/2$$

Statistical Evaluation. Student's *t*-test was used for statistical evaluation of differences in tumor size, animal body weight,

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[3] The abbreviations used are: FUra, 5-fluorouracil; dThd, thymidine.

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and WBC level between treated and control groups. Differences in mortality statistics between groups were compared for significance by χ^2 analysis. For all statistical evaluations, differences between groups with a statistical p of 0.05 or less were considered significant.

RESULTS

Correlation of Leukopenia and Mortality in Normal Female CD8F$_1$ Mice Treated with High-Dose dThd and FUra (Table 1). Ten normal, 3-month-old female CD8F$_1$ mice were treated with a toxic regimen of 3 weekly courses of dThd at 500 mg/kg followed 0.5 hour later by FUra at 75 mg/kg. WBC levels were measured immediately before the first course of treatment and immediately before the third course of treatment [*i.e.* day 14 (1 week after the second course of treatment)].

Leukopenia was evident in all mice on Day 14 (Table 1) and a direct correlation was observed between the magnitude of WBC depression on Day 14 and subsequent mortality by Day 21. With one exception (WBC count of 2225), those mice with WBC below 2625 on Day 14 were dead 1 week later, while all of the mice with WBC above 2775 were still alive on Day 21 (1 week after the third course of treatment).

Prevention of FUra-induced Toxicity with Depot Testosterone (Table 2). Table 2 records an experiment with the same toxic regimen of treatment with dThd and FUra given to each of 2 groups (Groups 1 and 2) of 10 normal female CD8F$_1$ mice. In addition, one of these groups (Group 2) received testosterone enanthate, 10 mg/mouse i.m., on the 2 successive days preceding each of the 3 weekly courses of dThd and FUra. Two additional groups of 10 mice each (Groups 3 and 4) received 3 weekly courses of a toxic dose (130 mg/kg) of FUra alone, and one of these 2 groups (Group 4) also was treated with testosterone enanthate on the 2 successive days preceding each of the 3 weekly courses of FUra. WBC levels were measured 5 days after the third course of treatment, and the mice were observed for mortality for an additional 21 days (Table 2).

Similar levels of toxicity [severe leukopenia, excessive body weight loss, and a high (50 to 70%) mortality] were observed in mice treated with FUra at 75 mg/kg in conjunction with dThd (Group 1) and in mice treated with FUra alone at 130 mg/kg (Group 3). (It should be noted that dThd alone at the dose and schedule used here produces no observable toxicity; data not shown). In contrast, the addition of testosterone to either FUra regimen (dThd and FUra, or FUra alone; Groups 2 and 4) resulted in a significant sparing effect on the WBC level (p, <0.005 for each of the 2 appropriate comparisons) and a substantial decrease in body weight loss. The amelioration of toxicity was also reflected in decreased mortality rates. In contrast to the 70% mortality in mice that received dThd and FUra without testosterone protection (Group 1), no mortality was observed in the testosterone-treated mice that received dThd and FUra (p, <0.01; Group 2). In mice that received FUra alone at 130 mg/kg, testosterone treatment resulted in a decrease in mortality rate from 50% to 20%. Although the latter difference is not significant in this particular experiment, over 20 repetitions of both types of experiments confirmed the protective effect of testosterone under these conditions.

Because fluid retention can be a complication of testosterone treatment, mice were observed carefully for signs of edema. There was no evidence of ascites or swollen extremities.

Comparison of Toxicity of dThd and FUra in Male and Female CD8F$_1$ Mice (Chart 1). In light of the fact that androgen treatment resulted in significant amelioration of the toxicity associated with FUra in female CD8F$_1$ mice, it seemed possible that there would be a sex-dependent difference in the toxic manifestations of FUra. Accordingly, we examined the toxicity of 2 weekly injections of dThd and FUra (at the same toxic dosages used in the preceding experiment) in 5-month-old male and female CD8F$_1$ mice and in 5-month-old male mice that had been castrated 17 days before administration of drugs. Average body weight changes in these 3 groups of mice during a 28-day observation period are plotted in Chart 1. The weight loss pattern was substantially different between normal males and females. Maximum weight loss in males was less than 10% as compared to nearly 29% in females. In addition, males returned to pretreatment weight quickly after the first injection of dThd and FUra and again within 9 days after the second injection, whereas the females did not regain weight after the first injection and still had not returned to normal weight as long as 21 days after the second injection. Thirty-three % (5 of 15) of the females were dead by Day 28, whereas there was no mortality (0 of 15) in the males. Weight loss was more profound in castrated males than in normal males. The castrated mice did not regain pretreatment weight level between the 2 courses of drug treatment, and weight recovery was much slower in castrated than in normal males after the second

Table 1

Correlation of depression of WBC level with mortality in female CD8F$_1$ mice treated with dThd (500 mg/kg) followed 0.5 hr later by FUra (75 mg/kg)

WBC/cu mm		Status of mouse on Day 21
Pretreatment	Day 14[a]	
10,650	850	Dead
10,850	1,275	Dead
9,925	1,850	Dead
11,850	2,200	Dead
6,075	2,225	Alive
11,100	2,625	Dead
16,175	2,775	Alive
7,725	3,000	Alive
13,575	3,375	Alive
10,925	3,750	Alive

[a] Day of third weekly course of drugs.

Table 2

Prevention of FUra-induced toxicity in female CD8F$_1$ mice by treatment with TE[a]

Treatment[b]	WBC/cu mm[c]	% of body wt loss at nadir	% of mortality[d]
1. $dThd_{500}$—0.5 hr→$FUra_{75}$	2406 ± 522[e]	27	70
2. TE^{10}—24 hr→TE^{10}—24 hr→$dThd_{500}$—0.5 hr→$FUra_{75}$	5369 ± 574	11	0
3. $FUra_{130}$	2263 ± 282	24	50
4. TE^{10}—24 hr→TE^{10}—24 hr→$FUra_{130}$	4838 ± 626	7	20

[a] TE, testosterone enanthate.
[b] Three courses of treatment were administered at weekly intervals with 10 mice/group. Subscripts, dose in mg/kg; superscripts, total dose, *i.e.*, mg/mouse.
[c] WBC levels were measured 5 days after the third course of treatment.
[d] Mice were observed for 3 weeks after the third course of treatment.
[e] Mean ± S.E.

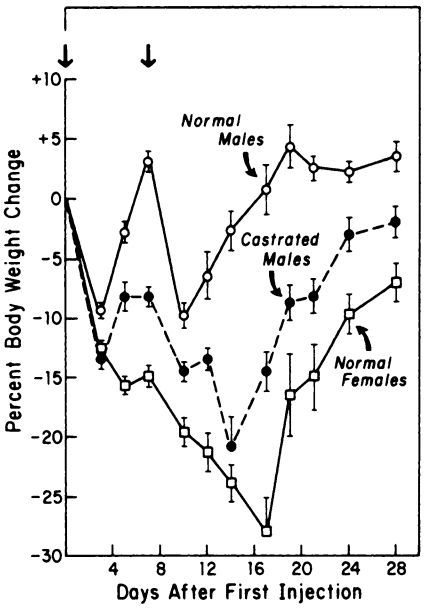

Chart 1. Body weight changes in normal male CD8F$_1$ mice, castrated male CD8F$_1$ mice, and normal female CD8F$_1$ mice treated with FUra at 75 mg/kg 0.5 hr after dThd at 500 mg/kg on Day 0 and Day 7 (drug injections, *arrows*). *Bars*, S.E. with 15 mice/group.

injection of FUra. Further, there was a 19% mortality (3 of 16) in the castrated males as compared to no mortality (0 of 15) in normal males.

Antitumor Activity of FUra in Testosterone-treated Mice (Chart 2). CD8F$_1$ mice bearing autochthonous breast tumors (averaging 200 to 300 mg) were used to determine the effect of testosterone on the antitumor activity of FUra. One group of tumor-bearing mice was treated with a regimen of 4 weekly injections of a known effective, but suboptimal dose of FUra alone (75 mg/kg). The second group received the same regimen of FUra, but in addition, it received testosterone enanthate (10 mg/mouse i.m.) on each of the 2 successive days preceding each injection of FUra. (There was no drug mortality in either of these groups. At its nadir, body weight loss was 12% in mice treated with FUra alone and 7% in mice treated with testosterone plus FUra.) Tumor weights throughout a 28-day observation period in the 2 FUra-treated groups as well as in controls treated only with 0.85% NaCl solution are shown in Chart 2. Statistically significant inhibition of tumor growth was observed in FUra-treated mice throughout the observation period. Tumor growth in the group treated with testosterone and FUra was essentially identical to that observed in the group treated only with FUra. The lack of effect of testosterone on the activity of FUra against autochthonous CD8F$_1$ breast tumors was confirmed in a separate experiment with FUra, 100 mg/kg (data not shown). These results illuminate a differential between tumor and host tissue in the protective action of testosterone.

Effect of Time of Administration of Depot Testosterone (Table 3). Table 3 presents an experiment with one course of testosterone enanthate (10 mg/mouse i.m. on each of 2 successive days) given to groups of normal female CD8F$_1$ mice beginning either 3 (Group 3) or 9 (Group 4) days before administration of a single 300 mg/kg dose of FUra. Control groups were treated with 0.85% NaCl solution (Group 1) or with FUra only (Group 2). WBC levels were measured on Day 0 (day of administration of FUra) and on Days 4, 7, and 11.

On Day 0, mice pretreated with depot testosterone beginning 9 days earlier (Group 4) showed slight elevation of WBC level compared to that of controls, whereas mice pretreated with testosterone only 3 days earlier (Group 3) had essentially the same WBC as did the controls. In Group 2, FUra-induced WBC depression was detectable on Day 4, reached a nadir of 2200 on Day 7 and was diminishing in those mice surviving the FUra

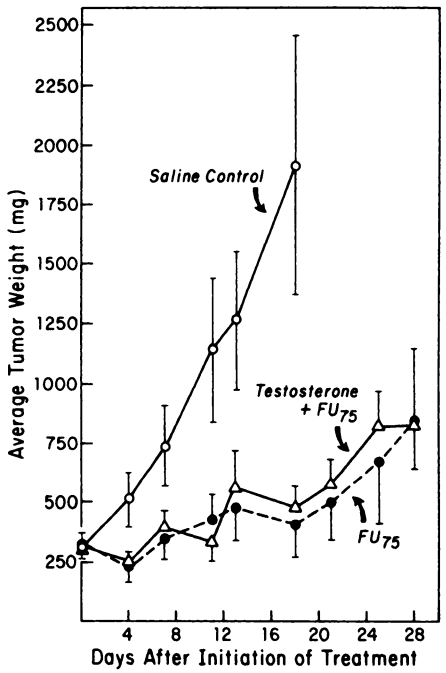

Chart 2. Antitumor activity of FUra in testosterone-treated, and untreated female CD8F$_1$ mice (15/group) bearing autochthonous mammary carcinomas. Saline control, 0.85% NaCl solution (○); FUra at 75 mg/kg i.p. on Days 0, 7, 14, and 21 (●); testosterone enanthate at 10 mg/mouse i.m. on 2 successive days preceding each dose of FUra at 75 mg/kg on Days 0, 7, 14, and 21 (△). *Bars*, SE.

Table 3

Protection against FUra-induced toxicity in female CD8F$_1$ mice is dependent upon time of administration of TE[a]

Treatment[b]	WBC/cu mm[c]				Dead/total on Day 14
	Day 0	Day 4	Day 7	Day 11	
1. Control (0.85% NaCl solution)	16,150 ± 621	17,340 ± 795	15,260 ± 772	16,770 ± 830	0/10
2. $FUra_{300}$	16,260 ± 889	7,030 ± 362	2,200 ± 200	6,200 ± 645	5/10
3. TE qid×2—24 hr→$FUra_{300}$	17,170 ± 898	6,880 ± 184	2,210 ± 327	6,600 ± 401	8/10
4. TE qid×2—8 days→$FUra_{300}$	20,830 ± 978	8,150 ± 420	4,540 ± 356	14,000 ± 1301	0/10

[a] TE, testosterone enanthate; qid×2, treatment once daily on each of 2 successive days.
[b] TE at 10 mg/mouse, i.m.; $FUra_{300}$ administered as a single dose of 300 mg/kg, i.p. (10 mice/group).
[c] Mean ± S.E.

treatment (6200) on Day 11. Fifty % of these mice (5 of 10) were dead on Day 14. No protection against FUra-induced toxicity was observed in mice pretreated with testosterone beginning 3 days before FUra (Group 3). These mice showed WBC nadir of 2210 on Day 7, a partial recovery (6660) in surviving mice on Day 11, and 80% (8 of 10) mortality by day 14. In contrast, mice treated with testosterone beginning 9 days before FUra (Group 4) showed a nadir WBC count on Day 7 of 4540 which was significantly higher (p, 2.83×10^{-4}) than that seen in FUra-treated mice that did not receive testosterone (Group 2). Further, recovery in Group 4 was more complete on Day 11 (WBC levels of 14,000; p, 5.72×10^{-3} *versus* Group 2). The testosterone-associated protection from leukopenia in mice treated with testosterone beginning 9 days before FUra also was correlated with decreased mortality (0% in Group 4 *versus* 50%, in Group 2; p, <0.05).

These results indicate that (with the depot form of testosterone) there is a time lag after administration of testosterone before the protective activity is manifest. In this experiment, it was demonstrated that significant protection could be obtained when testosterone treatment was initiated 9 days before, but not 3 days before, FUra. In subsequent confirmatory experiments (not shown), it was demonstrated that significant protection could be obtained when testosterone treatment was initiated 9, or 5 days before, but not 3 days before, FUra.

Optimal Dose of Depot Testosterone (Table 4). Table 4 presents the pooled results of 2 separate experiments with normal 3-month-old female $CD8F_1$ mice (10 and 15 mice/group in the first and second experiment, respectively) pretreated with one of varying concentrations of depot testosterone 5 days before each of 2 weekly courses of a toxic dose of dThd and FUra (*i.e.*, with FUra at 75 mg/kg). As a control, in each experiment, one group of mice received the same dThd-FUra treatment but was not pretreated with testosterone. WBC levels and body weights were measured 1 week after the second course of chemotherapy in each experiment (*i.e.*, the usual time, previously determined, at which nadir WBC levels were reached after this schedule of chemotherapy).

The dThd-FUra regimen used was clearly toxic (Group 1), yielding an average body weight loss of 23% and an average WBC count of 3672 (compared with an average pretreatment WBC count of 16,596, not shown), and ultimately, 18 of the 25 mice that received dThd and FUra without depot testosterone died within 3 weeks after the second course of drug treatment. Groups 2 to 4 demonstrate that depot testosterone treatment prior to each of 2 courses of the drugs resulted in a clear-cut dose-related protection against drug-induced toxicity. Doses of testosterone of 1 mg/mouse and above produced statistically significant protection against body weight loss (p, 3.8×10^{-6}), WBC depression (p, 4.49×10^{-7}), and mortality (p, 1.49×10^{-5}). The single lower dose of testosterone tested, 0.2 mg/mouse, had only a minimal protective effect which was not statistically significant in terms of body weight change or mortality, although protection against leukopenia was significant ($p = 0.002$) even at this low dose. At the highest dose of testosterone tested, 20 mg/mouse, there was essentially no weight loss (0.4%), the WBC count (15,596) was not significantly different from pretreatment count (16,596, not shown), and only 1 of 25 treated mice died. In terms of body weight loss and WBC count, results obtained with 20 mg/mouse were statistically superior to those obtained with the next highest dose of testosterone tested (5 mg/mouse). Therefore, a dose of 20 mg of testosterone per mouse is considered to be the optimal dose for protection in this murine system.

DISCUSSION

Leukopenia and body weight loss are manifestations of bone marrow and intestinal toxicity frequently associated with FUra therapy. The present results demonstrate a clear-cut ability of testosterone to protect the function of normal bone marrow and intestinal epithelium (*i.e.*, host weight) in female $CD8F_1$ mice against the toxicity of FUra. Further, testosterone did not interfere with the antitumor activity of FUra, and therefore, the net result was an increase in the operational antitumor specificity of FUra in testosterone-treated mice. Testosterone alone (data not shown) did not demonstrate antitumor activity against the hormonally independent $CD8F_1$ breast tumor (12). In ancillary studies (13, 14), we have demonstrated that the use of testosterone permitted the therapeutically beneficial combination of FUra with *N*-(phosphonacetyl)-L-aspartate (at doses which otherwise resulted in intolerable toxicity.

It is apparent from results in Chart 1 that androgens provide protection from FUra toxicity at "physiological" levels in the mouse. For example, normal male $CD8F_1$ mice exhibited less FUra-associated toxicity than did normal female $CD8F_1$ mice of the same age. Further, males were distinctly more susceptible to the toxic manifestations of FUra following castration. Nevertheless, when administered therapeutically, the optimal dose of testosterone enanthate for protection against FUra-induced toxicity (*i.e.*, leukopenia, body weight loss, and mortality) was found to be 20 mg per mouse. This dose is very similar to the

Table 4

Titration of TE[a] protection against FUra-induced toxicity in normal female $CD8F_1$ mice [pooled results from 2 separate experiments (10 and 15 mice/group)]

Treatment[b]	% of body wt change (Day 14)[c]	WBC/cu mm (Day 14)[c]	Mortality[d] (dead/total)
1. $dThd_{500}$—0.5 hr→$FUra_{75}$	−23.1	3,672	18/25
2. TE^{20}—5 days→$dThd_{500}$—0.5 hr→$FUra_{75}$	−0.4	15,370	1/25
3. TE^{10}—5 days→$dThd_{500}$—0.5 hr→$FUra_{75}$	−6.9	10,312	2/25
4. TE^{1}—5 days→$dThd_{500}$—0.5 hr→$FUra_{75}$	−12.4	9,188	2/25
5. $TE^{0.2}$—5 days→$dThd_{500}$—0.5 hr→$FUra_{75}$	−20.4	5,836	14/25

[a] TE, testosterone enanthate.
[b] Two courses of treatment were administered with a 1-week interval between courses. Subscripts and superscripts as defined in Table 2, Footnote *b*.
[c] Day 14 is 1 week after the second course of treatment.
[d] Mice were observed for mortality for 3 weeks after the second course of treatment.

reported optimal dose of testosterone (18 mg) required for stimulation of erythropoiesis in mice (16).

Host-sparing activity was increased substantially when the time interval between the administration of depot testosterone and FUra was increased from 2 days to 5 or 9 days. A similar lag period also has been reported for erythropoietic stimulation by testosterone in mice (16). We have not yet determined either the duration of the protective effect after a single administration of depot testosterone or that following daily administration of short-acting testosterone (*e.g.*, testosterone propionate). Such information will be necessary for planned studies where testosterone will be administered repeatedly during prolonged FUra chemotherapy treatment schedules.

The beneficial effect of androgens on erythropoieses in patients with anemic disorders is well established (8, 11). However, compelling evidence for androgen stimulation of granulopoiesis has accumulated only recently. Nortestosterone decanoate has been reported to stimulate granulopoiesis in mice made neutropenic by 1,3-bis(2-chloroethyl)-1-nitrosourea (27, 28). Kinetic analysis revealed androgen-stimulated proliferation of both the pluripotential marrow stem cell and its direct descendant, the unipotential stem cell (*i.e.*, the direct antecedent of the myeloblast) (27). Testosterone has also been shown to increase granulocyte colony formation in cultures of human marrow when testosterone was administered *in vitro* (19) or *in vivo* for several days before marrow collection (5). Here, too, the unipotential stem cell was implicated as the target for testosterone stimulation (26).

In addition to the stimulation of bone marrow stem cells (18), testosterone may provide some specific biochemical protection in relation to FUra-induced injury in intestinal epithelium and bone marrow. Since we believe that the major biological activity of FUra ensues as a result of its incorporation into RNA (13–15, 21), it is pertinent to note that testosterone has been reported to affect RNA biosynthesis; testosterone has also been shown to increase RNA polymerase activity in the livers of immature rats and castrated mice (1, 6). Further, Tarnowski and Zak (25) reported increased aspartate transcarbamoylase activity in the liver and kidney of castrated rats. We are currently looking at the effect of FUra on RNA biosynthesis in control and testosterone-treated mice. It is possible that the selective protection of the host provided by testosterone may be due in part to differential activity in normal as opposed to tumor tissue as regards pyrimidine and RNA metabolism.

Androgenic hormones long have been utilized clinically as specific anticancer therapy for hormone-responsive cancers (20, 24). In addition, anabolic steroids are increasingly recommended as adjuvants to chemotherapy in cancer patients for the general purpose of improving erythropoietic parameters, nutritional intake, weight gain, and performance status (4, 7–9, 22). In cancer patients, androgens have been reported to protect against leukopenia induced by FUra (3, 4) or by the combination of vincristine, Adriamycin, and cyclophosphamide (10). However, the effect of androgen treatment on the antitumor efficacy was not determined in these clinical studies. The most promising results have been reported in a recent study of the addition of testosterone to a maintenance regimen of Cytoxan, methotrexate, and FUra in breast cancer patients (26) where significant increase in duration of partial remission was ascribed to greater drug delivery permitted due to increased marrow support by the androgen.

Because of the small differential in cytotoxic susceptibility between normal and neoplastic tissue, host toxicity is the success-limiting factor in current cancer chemotherapy. The ability to reduce the host toxicity associated with as important a clinical agent as FUra with testosterone without loss of antitumor effect (demonstrated in the present study) would appear to provide the basis for a significant advance in cancer chemotherapy with FUra or with FUra-containing combinations of chemotherapeutic drugs.

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