Time Dependence for the Protective Effect of Androgen from Procarbazine-induced Damage to Rat Spermatogenesis

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ABSTRACT

The protective effects of androgen pretreatment on the procarbazine-induced killing of spermatogonial stem cells in Wistar rats have been investigated. Using testosterone-filled Silastic implants (200 mm²) the degree of protection from four weekly doses of procarbazine (100 mg/kg) was found to be dependent upon the androgen pretreatment time interval as assessed by quantitative histology. No protective effect was seen until rats had received 4 wk of pretreatment with androgen, whereafter protection increased to a maximum (about 20 to 30% of tubule cross-sections exhibiting recovery) after 8 to 12 wk of pretreatment. In contrast, the same level of maximal protection could be obtained by 6 wk of pretreatment using testosterone enanthate, suggesting that differences in protection may be achieved using different modes of androgen administration.

INTRODUCTION

The use of chemotherapy in the treatment of malignant disease has met with varying degrees of success. Most notable of the successful therapeutic protocols are those used in the treatment of lymphomas and testicular tumors. While the protocols have developed, there has been a remarkable improvement in remission for these diseases, many of the patients are rendered sterile (1-4). As many of these patients are of reproductive age this has given rise to a requirement of attempt protection of the spermatogonial stem cells during therapy.

One strategy that has been suggested is to interrupt the pituitary secretion of gonadotrophins, reducing the trophic support to the testis and thus spermatogonial cells, which may be rendered quiescent. The resting stem cells may thus become relatively resistant to cytotoxic drugs. A number of studies in various species have used this approach to produce qualitative evidence for such protection (5-8). More recently we have demonstrated (9), using an androgen pretreatment, a quantitative and reproducible protection of the spermatogonial stem cells in the rat against severe damage induced by multiple treatments with the methyl hydrazine derivative PCB. In this paper we describe the extension of these studies to investigate the dependence of this effect on both the mode and pretreatment administration time of androgen.

MATERIALS AND METHODS

Animals and Drugs

Inbred Wistar rats obtained from the Medical Research Council, Mill Hill, London, United Kingdom, were used at 10 to 12 wk (250 to 300 g of body weight) of age. They were housed in standard conditions and were allowed food and water ad libitum. Procarbazine hydrochloride was obtained as a gift from Roche Products. Testosterone and testosterone enanthate were obtained from Sigma Chemical Co., Poole, Dorset, United Kingdom.

Experimental Protocols

In the previous study, androgen was administered as three injections per wk of testosterone enanthate at 240 µg/100 g of body weight (9). For the present study, it was decided to investigate both of the mode of administration and the pretreatment time interval. Therefore androgen was administered using a silastic tubing system (10). Implants were constructed from Silastic tubing (medical grade; Dow Corning, Ltd., MI; Catalogue No. 602-305; inner diameter, 0.195 cm; outer diameter, 0.3125 cm) and sealed with medical adhesive silicone type A (Dow Corning, Catalogue No. 891). From preliminary experiments in which a number of androgen esters were investigated, it was found that, in our rats, testosterone itself proved to be the most effective suppressive agent. A testosterone implant of 200 mm² (2 cm in length using the above tubing; release rate, 40 µg/day) was effective in reducing serum LH concentrations, testis weight, and testicular sperm head counts.

Group 1. These rats received testosterone implants and saline injections at 4 wk after the last PCB/saline injection. The Silastic implants were removed and carefully examined for leaks, under ether anesthesia, from the rats. All animals were killed at 8 wk after the final dose of PCB or saline. It was judged that at this time any spermatogonial cells would be derived from surviving stem cells.

In a further experiment, four extra groups of eight rats received an exact repeat of the original protocol devised for the original testosterone enanthate study (9). These rats were all killed at 8 wk after the final dose of PCB or saline.

At the appropriate time interval the rats were bled by cardiac puncture under ether anesthesia and were killed by overdosing with the anesthetic. The blood was allowed to clot, and serum was prepared and stored at −20°C until used in the hormone assays. Testes, epididymides, ventral prostate, and seminal vesicles were all removed from the rats and weighed wet. The tests were fixed for histology in Zenker-formol.

ASSAY PROCEDURES

Hormones. Serum concentrations of LH and FSH were estimated by double antibody radioimmunoassay as described previously (9) using antiserum (rLH-S-7, rFSH-S-11), purified tracer (rLH-I-6, rFSH-I-6), and reference preparations (rLH-RP-2, rFSH-RP-2) provided by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. Each sample was assayed at least in duplicate (usually in triplicate) using volumes of 200 µl for LH and 100 µl for FSH. Assay sensitivities were 0.4 ng/ml for LH and 3.0 ng/ml for FSH. Coefficients of variation within the assays were 14% for LH and 10% for FSH.

Histology. Survival of spermatogonial stem cells was assessed by the tubule regeneration assay as described previously (9). Counts of regenerating and empty cross-sections were made in two testicular cross-sections per rat. The mean count for each rat was taken, and these were...
used to calculate the overall mean for the groups. As observed previously, a background of empty tubule cross-sections (of about 10%) was observed in the controls, and this was again corrected for as in our previous study (9).

Statistically significant changes were determined using a one-way analysis of variance, and if significant differences were indicated these were defined using the Student-Newman-Keuls multiple range test (11). Data from the counts of repopulating cross-sections were analyzed using the nonparametric Mann-Whitney U test as some of the data were defined using the Student-Newman-Keuls multiple range test (11).

Also reduced to this level in the arachis oil plus PCB group (testosterone enanthate experiment). In the groups which received combined treatments testis weights were reduced significantly following all pretreatment periods compared to both control groups. Furthermore, the reduction in testis weights in the testosterone plus PCB groups was to about the same value as that in the cholesterol plus PCB groups. In the testosterone enanthate experiment the reduction in testis weight in the testosterone plus PCB groups was significantly greater than in the cholesterol plus PCB groups. The changes observed in testis weight were the same even when they were expressed relative to body weight. Epididymal weights were also significantly reduced in both sets of PCB-treated groups when compared to either the androgen or cholesterol controls. Androgen pretreatment generally had little effect on epididymal weight 8 wk after implant removal. Ventral prostatic weight (Table 2) was unchanged by any of the treatments used, as was seminal vesicle weight (data not shown).

Fig. 2 shows the percentage of seminiferous tubule cross-sections found to be repopulating at 8 wk after the final dose of PCB, as a function of the pretreatment time interval for the two PCB-treated groups. In rats treated with cholesterol implants plus PCB severe damage to spermatogenesis was observed in all groups, with consistently no repopulation of the

<p>| Table 2 Ventral prostatic weights (g) 8 wk after the final PCB or saline treatments |
|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|</p>
<table>
<thead>
<tr>
<th>Pretreatment time</th>
<th>Treatment group</th>
<th>T* + PCB</th>
<th>T + S</th>
<th>CHL + PCB</th>
<th>CHL + S</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 h</td>
<td>T* + PCB</td>
<td>0.41 ± 0.02*</td>
<td>0.49 ± 0.01</td>
<td>0.43 ± 0.01</td>
<td>0.45 ± 0.02</td>
</tr>
<tr>
<td>1 wk</td>
<td>T* + PCB</td>
<td>0.46 ± 0.02</td>
<td>0.50 ± 0.03</td>
<td>0.44 ± 0.03</td>
<td>0.54 ± 0.04</td>
</tr>
<tr>
<td>2 wk</td>
<td>T* + PCB</td>
<td>0.50 ± 0.04</td>
<td>0.53 ± 0.03</td>
<td>0.48 ± 0.02</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>4 wk</td>
<td>T* + PCB</td>
<td>0.49 ± 0.02</td>
<td>0.49 ± 0.02</td>
<td>0.46 ± 0.01</td>
<td>0.57 ± 0.04</td>
</tr>
<tr>
<td>6 wk</td>
<td>T* + PCB</td>
<td>0.54 ± 0.03</td>
<td>0.54 ± 0.03</td>
<td>0.48 ± 0.02</td>
<td>0.54 ± 0.02</td>
</tr>
<tr>
<td>8 wk</td>
<td>T* + PCB</td>
<td>0.48 ± 0.02</td>
<td>0.54 ± 0.03</td>
<td>0.45 ± 0.03</td>
<td>0.49 ± 0.04</td>
</tr>
<tr>
<td>10 wk</td>
<td>T* + PCB</td>
<td>0.51 ± 0.03</td>
<td>0.52 ± 0.05</td>
<td>0.45 ± 0.01</td>
<td>0.54 ± 0.03</td>
</tr>
<tr>
<td>12 wk</td>
<td>T* + PCB</td>
<td>0.49 ± 0.04</td>
<td>0.51 ± 0.03</td>
<td>0.46 ± 0.02</td>
<td>0.49 ± 0.02</td>
</tr>
<tr>
<td>6 wk*</td>
<td>T* + PCB</td>
<td>0.52 ± 0.02</td>
<td>0.57 ± 0.04</td>
<td>0.48 ± 0.04</td>
<td>0.59 ± 0.04</td>
</tr>
</tbody>
</table>

* T, testosterone; PCB, procarbazine hydrochloride; S, saline; CHL, cholesterol; TE, testosterone enanthate; AO, arachis oil.

Statistically significant changes were determined using a one-way analysis of variance, and if significant differences were indicated these were defined using the Student-Newman-Keuls multiple range test (11). Data from the counts of repopulating cross-sections were analyzed using the nonparametric Mann-Whitney U test as some of the data (cholesterol plus PCB) were not normally distributed.

RESULTS

No difference was observed within treatment groups in either testis or epididymal weights as a function of the pretreatment time intervals, and thus all data were pooled to give overall mean values, which are given in Table 1. Testicular weight had recovered to control values in all of the groups treated with testosterone plus saline. Cholesterol plus PCB treatment resulted in a significant reduction of testis weight to about 40 to 50% of the value obtained from controls. The testis weight was
epithelium. In contrast, pretreatment with androgen produced a protective effect on spermatogonial stem cells which was clearly dependent upon the pretreatment time interval. The maximal protective effect with testosterone implants was observed by 8 wk of pretreatment and was not increased by extending this time interval. The amount of protection observed was variable between rats within a given pretreatment time interval as indicated by the large standard errors. For instance, the percentage of repopulation observed for 8, 10, or 12 wk of pretreatment ranged from 10 to 81%, 7 to 45%, and 1 to 50%, respectively.

Pretreatment of rats with testosterone enanthate for 6 wk also resulted in a protective effect which was significantly greater ($P < 0.01$) when compared to 6 wk of pretreatment with testosterone implants. The level of protection observed with testosterone enanthate pretreatment was the same as that observed for the longer (8 wk or more) implant pretreatment times. No effect was observed on the percentage of tubules repopulating when rats were treated with androgen only (data not shown).

Serum LH, as a function of the pretreatment time interval, 8 wk after the final PCB/saline injection is shown in Fig. 3. No changes, as compared with the levels observed in the cholesterol overall controls, were seen when rats were treated with androgen alone. The LH changes in the PCB-treated groups were more variable. Rats treated with cholesterol plus PCB had elevated serum LH levels, when compared to both cholesterol and androgen controls, for all pretreatment time intervals except 4 wk. In contrast, LH was generally unchanged in those rats treated with testosterone plus PCB. The exceptions to this were the 2- and 12-wk pretreatment groups where serum LH was significantly elevated. Serum LH was unchanged by pretreatment with testosterone enanthate but was significantly elevated in both of the PCB-treated groups for this experiment.

The changes observed in serum FSH (Fig. 4) were more clear. Treatment with androgen alone had no effect on serum FSH levels. In all PCB-treated groups, however, serum FSH demonstrated a consistent significant elevation compared to both control groups.

**DISCUSSION**

In a previous study we have demonstrated that it is possible to protect spermatogonial stem cells from severe PCB-induced damage (9). We have now demonstrated that this protection depends upon both the androgen pretreatment time interval and the mode of steroid administration.

Pretreatment of rats in this study with testosterone-filled Silastic capsules where androgen output was sufficient to suppress spermatogenesis did not result in any permanent damage to the testis or pituitary, even when the treatment period was as long as 16 wk (12 wk of pretreatment plus 4 wk of continued treatment during saline administration). This lack of long-term damage was indicated by the recovery of testis and epididymal weights and serum levels of pituitary gonadotrophins to the values seen in controls.

Treatment with cholesterol plus PCB resulted in severe damage to the seminiferous epithelium with complete destruction of the spermatogonial stem cell compartment, which is in line with previous observations (6, 9). Furthermore, from the increase in serum concentrations of LH, there appeared to be some indication that damage to the Leydig cell population had been induced, which is also consistent with our previous observations (9). The lack of change in weights of the androgen-dependent organs, however, indicates that the compensatory increase in serum LH was sufficient to maintain adequate androgen secretion. Therefore it is clear that Leydig cells function adequately even without androgen protection. The general lack of change in the serum levels of LH in the combined treatment groups contrasts with the elevations seen with PCB alone. This may be indicative of a fairly normal Leydig cell function following combined treatment, suggesting that the androgen pretreatment may protect this cell type from PCB-induced damage. If this were the case, however, then this should also have been expected for those animals pretreated with testosterone enanthate, which was not seen. Therefore no clearly consistent explanation can be given for these results. The increase in serum FSH observed after PCB treatment is again consistent with previous observations (9) and probably reflects the damage to the seminiferous epithelium. Similar increases in serum FSH were seen in the combined treatment groups as compared to the "PCB-only" groups. This, together with the similar reductions in testis and epididymal weights, suggests equivalent degrees of damage to the seminiferous epithelium. However, the quantitative histological observations did not confirm this impression.

It was clear from the histological data that pretreatment of rats with androgen had a protective effect against the PCB-induced spermatogonial stem cell killing. There are three points of particular interest in these observations. (a) It is clear that, for the implant system used, at least 6 wk are required to obtain some protection, while 8 wk or more produce a maximal effect. Therefore from the data we conclude that the minimum pre-

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**Fig. 3.** Serum LH concentrations (rLH-RP-2) as a function of the cholesterol/testosterone pretreatment time interval, before 4 weekly doses of procarbazine hydrochloride, at 8 wk after the final drug dose. Points, mean of determinations from 8 rats/group; bars, SE. T, testosterone implant; C, cholesterol implant; SAL, saline; TE, testosterone enanthate; AO, arachis oil. *P < 0.01 versus all controls.

**Fig. 4.** Serum FSH concentrations (rFSH-RP-2) as a function of the cholesterol/testosterone pretreatment time interval, before 4 weekly doses of procarbazine hydrochloride, at 8 wk after the final drug dose. Points, mean of determinations from 8 rats/group; bars, SE. T, testosterone implant; C, cholesterol implant; PCB, procarbazine; S, saline; TE, testosterone enanthate; AO, arachis oil. All PCB-treated groups had significantly elevated ($P < 0.001$) levels of FSH when compared to the control groups.
As this hormone influences Sertoli cell function (12, 13), and the reason for this is not at all clear. Both administration protocols used produced approximately the same levels of spermatogenic suppression as evaluated by testis weight and sperm head counts. In separate experiments we have found that the rates at which the gonadotrophins are suppressed following implantation or injection are similar (Fig. 5). Taken together, these results suggest that there is no fundamental difference in the suppressive abilities of these two protocols. Thus no obvious reason exists to explain these time-related differences for the protective effects. One possibility may be that FSH appears to be less well suppressed in the implanted rats (Fig. 1) at 4 wk. The results of the present study may have implications for the application of such approaches to clinical situations. As can be clearly seen, the mode of suppressive agent administration may be important in obtaining protection. This is particularly true when the influence of pretreatment suppression time is considered. For most centers the maximum time from presentation of a patient with Hodgkin’s disease to the commencement of treatment would be of the order of 3 to 4 wk. Thus, it is indicated from the present data that any protective measures using such endocrine manipulations should begin immediately at presentation. The mode of administration may therefore become important in achieving as rapid a protective effect as possible.

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