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Effect of androgen therapy on survival and suppressor cell activity in aged NZB/NZW F_1 hybrid mice

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SUMMARY

Male NZB/NZW F_1 hybrid (B/W) mice survive their first year of life and die of lupus nephritis or lymphoid malignancy during the second year. Androgen therapy, even if delayed until 9 months of age, improves survival considerably. We report here that androgen therapy in aged B/W mice is associated with improved cell-mediated immune function as well as increased survival. Androgen treated mice have significantly augmented spleen cell responses to phytohaemagglutinin (PHA) and a decreased incidence of abnormal splenic suppressor activity. These results suggest that androgen may prolong survival in B/W mice in part through an effect on abnormally suppressive regulatory cells that impair T lymphocyte function.

INTRODUCTION

NZB/NZW F_1 hybrid (B/W) mice are a model of spontaneously occurring autoimmune disease and lymphoid malignancy. Female mice develop severe lupus nephritis at about 6 months of age and essentially all die by 1 year of age. Male mice survive their first year and die with nephritis or malignancy during the second year (Talal & Steinberg, 1974). Sex hormones, particularly androgens, play a role in the onset and severity of nephritis; castrated males have a female pattern of survival and androgen treated females live much longer than controls (Roubinian, Papion & Talal, 1977; Roubinian *et al.*, 1978, 1979).

A concomitant to the development of autoimmunity in older male B/W mice is a severe immunodeficiency state. These animals have marked impairment of cell-mediated immunity that is out of proportion to the change in Thy-1 bearing T cells. This immunodeficiency is due, in part, to the occurrence of splenic suppressor activity with particularly strong effects on phytohaemagglutinin (PHA) responsive T lymphocytes. The suppression is mediated by cells that appear to be small lymphocytes. They are non-phagocytic, non-adherent to nylon wool and resistant to anti-Thy-1 and complement treatment (Michalski, McCombs & Talal, 1979). A T cell lineage is suggested by the ablation of suppressor activity with anti-Ly 1·2 antiserum and complement (Michalski *et al.*, 1980).

We report here the results of a study of androgen therapy and suppressor cell activity in old male B/W mice. A single implant of androgen at 9 months of age was associated with strikingly improved survival. This clinical improvement was accompanied by improved T lymphocyte function and associated with a greatly decreased incidence of suppressor activity in the spleens of treated mice.

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MATERIALS AND METHODS

Mice. B/W mice were obtained from the vivarium of the University of California, San Francisco. All animals were aged at the San Francisco Veterans Administration animal facility.

Study protocol. Twenty-eight male B/W mice 9 months of age were randomly assigned to a treatment group of 14 mice that received a silastic tube implant containing 6 mg of androgen (5-α-dihydrotestosterone) or a control group receiving a sham implant. This procedure results in the slow release of physiological levels of testosterone for 6–8 months (Roubinian *et al.*, 1978).

The operated animals were observed twice weekly for mortality and after 9 months the surviving mice were sacrificed for the *in vitro* studies described below. Because of the small number of surviving sham treated animals, the data from a similarly aged non-operated control group were compared with those of the two operated groups. These control mice were tested at about the same time using the same procedures and reagents (Michalski *et al.*, 1979).

Spleen cell preparation and fractionation. Mice were killed by cervical dislocation and the spleens removed aseptically. Single cell suspensions were prepared by gently teasing the spleens in medium and allowing clumps to settle out.

Culture conditions. All cultures were done in 0·2 ml total volume in flat-bottom wells of plastic microculture plates (Linbro Scientific, Inc., Hamden, Connecticut, USA, No. 1S-FB-96TC). Each well contained a total of 5×10^5 viable spleen cells suspended in a final concentration of 10% heat-inactivated fetal calf serum (FCS) (GIBCO, New York, USA), 1% antibiotic antimycotic, and 1% L-glutamine in RPMI 1640 medium (Pacific Biological Co., Berkeley, California, USA) buffered with 10 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid (HEPES). The final concentration of purified PHA (lot No. K 1794) (Burroughs Wellcome, Beckenham, UK) was 1 μg/ml, a suboptimal concentration that results in a late peak of ^{3}H-thymidine incorporation (Michalski & McCombs, 1977). Seventy-two hours after 6-h labelling with 1 μCi tritiated thymidine (3 Ci/mmol, Schwarz-Mann, Orangeburg, New York), cultures were harvested onto glass fibre filters with a multiple automated sample harvester (Otto Hiller, Madison, Wisconsin, USA).

The filters were air dried, placed in scintillation vials with a toluene-Liquifluor (New England Nuclear, Boston, Massachusetts, USA) cocktail, and counted in a Packard scintillation counter. The data from quadruplicate cultures were analysed on a Wang computer programmed to exclude individual data more than 3 standard deviations from the calculated means. Data were expressed as net ct/min (stimulated ct/min minus unstimulated ct/min). Unstimulated counts were 633 ± 144 for androgen treated mice and 586 ± 73 ($\bar{X} \pm$s.e. (mean)) for control animals. Seven of 10 androgen treated mice and seven of 14 controls had a stimulated response which was more than two-fold greater than background.

Mixing protocol to determine suppression of mitogen-induced lymphocyte activation. Five hundred thousand control young (3–4 months) B/W or presumptive suppressor cell containing old B/W spleen cells were cultured with or without mitogen, and thymidine incorporation was measured. These same cell preparations were mixed in wells ($2{\cdot}5 \times 10^5$ of each, to give the same initial cell density) and the response of the cell mixture was determined and compared with the expected response.

Calculation of percentage suppression.

$$\% \text{ Suppression} = 1 - \frac{\text{observed ct/min of cell mixture}}{\text{expected response of cell mixture}} \times 100$$

where the expected ct/min of the cell mixture = (net ct/min of young spleen cells + net ct/min of old spleen cells) divided by 2.

Abnormal splenic suppressor activity was defined as the ability of the old B/W spleen cells to suppress greater than 50% of the expected response of the young B/W spleen cells.

RESULTS

Androgen therapy is associated with improved survival

Twenty-eight male B/W mice 9 months of age were randomly assigned to groups which received

either one subcutaneous implant of androgen (6 mg of 5-α-dihydrotestosterone) or a sham implant. The mice were observed twice weekly for mortality and the survivors were sacrificed after 9 months for *in vitro* assays of cellular immune function. Androgen therapy was found to be associated with markedly improved survival during the second year of life. At 18 months of age, 76% of the androgen treated animals were alive compared with only 33% of the controls (Fig. 1).

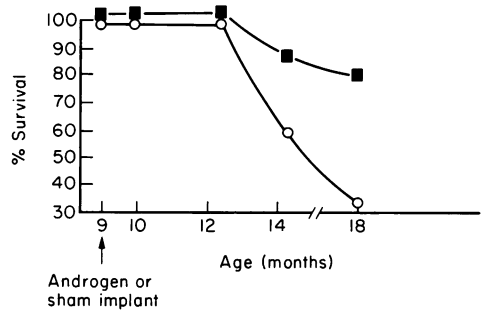

Fig. 1. Androgen therapy begun at 9 months of age improved survival in male B/W mice. Androgen implant (■——■), 76%; sham implant (○——○), 33%.

Androgen treatment augments cell-mediated immune function

Cell-mediated immune function was measured by determining the proliferative response of spleen cells to PHA. The reactivity of spleen cells from androgen treated mice was found to be significantly higher than that of the control groups (Table 1).

Table 1. Responsiveness to PHA in androgen treated and control NZB/NZW F_1 hybrid mice

Group	Number of mice	$\bar{x} \pm$ s.e.(mean)*	Geometric mean†
Androgen treated	10	$3{\cdot}1564 \pm 0{\cdot}1829$	1,434§
Controls‡	14	$2{\cdot}6794 \pm 0{\cdot}1284$	478

* $\log_{10}$ net ct/min ± standard error.
† Net ct/min.
‡ Four sham operated + 10 unoperated controls.
§ $t = 2{\cdot}138$; $P < 0{\cdot}03$.

Impaired spleen cell responses correlate with splenic suppressor activity

Suppressor activity against T cell responses of young syngeneic spleen cells was measured by mixing $2{\cdot}5 \times 10^5$ old B/W spleen cells with $2{\cdot}5 \times 10^5$ young spleen cells and comparing the response of the cell mixture to the expected response (See Materials and Methods). Linear regression analysis showed a highly significant correlation between the magnitude of suppression and the degree of impaired mitogen responsiveness (Fig. 2).

Androgen therapy is associated with a decreased incidence of abnormal splenic suppressor activity

Abnormal splenic suppressor activity was defined as the ability of the old B/W spleen cells to suppress greater than 50% of the expected response of the young B/W spleen cells. Only three of 10 androgen treated mice had abnormal splenic suppressor activity compared with 13 of 14 control animals ($P < 0{\cdot}0023$, by Fisher's Exact Test).

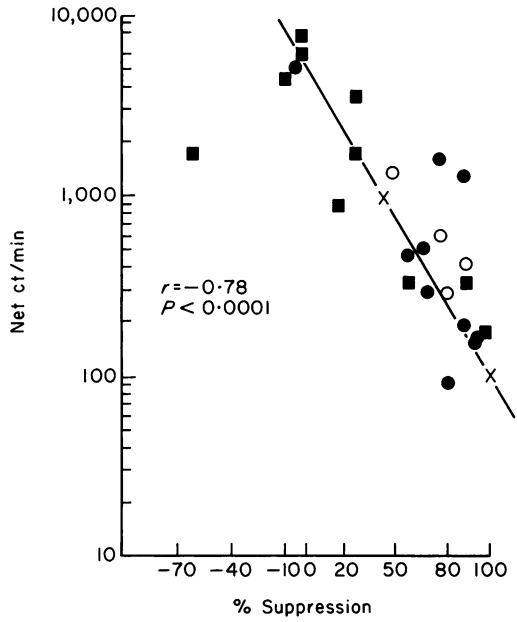

Fig. 2. There is a strong correlation between impaired PHA reactivity in old B/W mice and the ability of their spleen cells to suppress the response of young mice. ● = unoperated control mice; ○ = sham implant; ■ = androgen treated mice.

DISCUSSION

The purpose of this study was to shed light on the mechanism of action of androgen in the delayed treatment of B/W mice. We used aged male mice because they have a prolonged survival into their second year of life and develop severe immunodeficiency associated with suppressor cells that act on PHA responsive T cells (Michalski *et al.*, 1979). Suppressor cells such as these have been reported to occur early in the life of female B/W mice (Roder, Bell & Singhal, 1975, 1977) and may contribute to the pathogenesis of autoimmunity. In older mice this suppressor mechanism might contribute to worsening of autoimmunity or to death from infections and lymphoproliferative disease.

The correct interpretation of suppressive activity as being indicative of 'suppressor cell' function can be difficult. For example, cells that suppress under one set of circumstances (e.g., time of culture, cell number) may augment the same response under another set of conditions (Farrant & Newton, 1981). We have previously demonstrated that the regulatory activity of old B/W spleen cells is consistently suppressive over a fairly wide range of culture intervals, and ratios of old to young spleen cells (Michalski *et al.*, 1979). However, it was not possible to examine all the potentially important parameters and in this study animals were tested under only one set of conditions. Our interpretations of the suppressive activity of the old spleen cells as suppressor cell activity should be considered a tentative conclusion.

The precise nature and role of the putative suppressor cells in murine lupus is still unclear. They are lymphoid cells without the classic features of T cells, B cells, or macrophages. Since the suppressor activity is ablated by anti-Ly 1·2 alloantiserum and complement (Michalski *et al.*, 1980), the suppressor cells in the spleens of aged B/W mice are most likely to have a T cell lineage, but an abnormally low expression of Thy-1 antigen. Indeed, Gershon (1978) reported that B/W mice have unusually high percentages of Thy-1 negative, Ly-1 bearing spleen cells.

The studies presented here help to clarify the role of suppressor activity in the immunodeficiency of aged B/W mice and suggest that the therapeutic effect of androgen may result, in part, from effects on the immune system. The strong correlation between the severity of impaired cellular immunity (as measured by decreased PHA reactivity) and the degree of suppression of the response

of young B/W spleen cells is consistent with a major and primary contribution of the suppressor activity to the immunodeficiency state. As we have previously reported for delayed androgen therapy of female B/W mice (Roubinian *et al.*, 1979), a single implant of 6 mg of 5-α-dihydrotestosterone in male B/W mice at 9 months of age resulted in remarkedly increased survival time compared with that of age matched sham implanted controls. Moreover, in these studies we found that in treated mice compared with controls, spleen cell PHA reactivity was significantly improved and the incidence of abnormal splenic suppressor activity was greatly reduced. Thus, delayed androgen therapy in aged male B/W mice results in improved survival associated with improved cellular immune responses and reduced spleen cell suppressor activity.

These findings are consistent with the view that immunodeficiency in aged B/W mice contributes to mortality. We suggest that androgens may improve survival, in part, by improving cellular immune responses and that the improved immune reactivity may result from the effect of androgens on the generation or reactivity of splenic suppressor cells in aged mice. The studies of Eidenger & Garrett (1972) suggest that androgens modulate normal immune reactivity through effects on the thymus. The abnormal suppressor activity in old B/W mice is associated with an unusual Thy-1 negative, Ly 1·2 bearing lymphocyte (Gershon, 1978). These cells could result from disordered thymic maturation of T cells. Should this be the case, androgens may act by correcting the abnormal thymic maturation process.

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